ACS SYMPOSIUM SERIES 777

Pesticide Biotransformation in Plants and Microorganisms

Similarities and Divergences

EDITED BY

J. Christopher Hall, Robert E. Hoagland,
and Robert M. Zablotowicz

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.fw001

Pesticide Biotransformation in Plants and Microorganisms

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.fw001

ACS SYMPOSIUM SERIES 777

Pesticide Biotransformation in Plants and Microorganisms

Similarities and Divergences

J. Christopher Hall, EDITOR
University of Guelph

Robert E. Hoagland, EDITOR
Agricultural Research Service,
U.S. Department of Agriculture

Robert M. Zablotowicz, EDITOR
Agricultural Research Service,
U.S. Department of Agriculture

American Chemical Society, Washington, DC

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.fw001

Library of Congress Cataloging-in-Publication Data

Pesticide biotransformation in plants and microorganisms : similarities and divergences /
J. Christopher Hall, editor, Robert E. Hoagland, editor, Robert M. Zablotowicz, editor.
p. cm.—(ACS symposium series ; 777)

Includes bibliographical references.

ISBN 0-8412-3704-2

1. Pesticides—Metabolism—Congresses. 2. Pesticides—Metabolic detoxification—Congresses. 3. Microbial enzymes—Congresses. 4. Plant enzymes—Congresses.

I. Hall, J. Christopher, 1953- II. Hoagland, Robert E. III. Zablotowicz, Robert Michael. IV. American Chemical Society. Meeting (218th : 1999 : New Orleans, Louisiana) V. Series.

QP801.P38 P475 2000
572′.42—dc21 00-55873

The paper used in this publication meets the minimum requirements of American National Standard for Information Sciences—Permanence of Paper for Printed Library Materials, ANSI Z39.48-1984.

Copyright © 2001 American Chemical Society

Distributed by Oxford University Press

All Rights Reserved. Reprographic copying beyond that permitted by Sections 107 or 108 of the U.S. Copyright Act is allowed for internal use only, provided that a per-chapter fee of $20.00 plus $0.50 per page is paid to the Copyright Clearance Center, Inc., 222 Rosewood Drive, Danvers, MA 01923, USA. Republication or reproduction for sale of pages in this book is permitted only under license from ACS. Direct these and other permission requests to ACS Copyright Office, Publications Division, 1155 16th St., N.W., Washington, DC 20036.

The citation of trade names and/or names of manufacturers in this publication is not to be construed as an endorsement or as approval by ACS of the commercial products or services referenced herein; nor should the mere reference herein to any drawing, specification, chemical process, or other data be regarded as a license or as a conveyance of any right or permission to the holder, reader, or any other person or corporation, to manufacture, reproduce, use, or sell any patented invention or copyrighted work that may in any way be related thereto. Registered names, trademarks, etc., used in this publication, even without specific indication thereof, are not to be considered unprotected by law.

PRINTED IN THE UNITED STATES OF AMERICA

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.fw001

American Chemical Society
Library
1155 16th St., N.W.
Washington, D.C. 20036

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.fw001

Foreword

The ACS Symposium Series was first published in 1974 to provide a mechanism for publishing symposia quickly in book form. The purpose of the series is to publish timely, comprehensive books developed from ACS sponsored symposia based on current scientific research. Occasionally, books are developed from symposia sponsored by other organizations when the topic is of keen interest to the chemistry audience.

Before agreeing to publish a book, the proposed table of contents is reviewed for appropriate and comprehensive coverage and for interest to the audience. Some papers may be excluded in order to better focus the book; others may be added to provide comprehensiveness. When appropriate, overview or introductory chapters are added. Drafts of chapters are peer-reviewed prior to final acceptance or rejection, and manuscripts are prepared in camera-ready format.

As a rule, only original research papers and original review papers are included in the volumes. Verbatim reproductions of previously published papers are not accepted.

ACS Books Department

Dedication

This book is posthumously dedicated to Dr. Richard H. Shimabukuro, Research Plant Physiologist of the Agricultural Research Service, U.S. Department of Agriculture, who dedicated his career to understanding the metabolism of pesticides in plants. His pioneering research has been an inspiration to students and colleagues and has provided fundamental pathways.

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.fw001

Preface

The basis for selectivity of plants and microorganisms to xenobiotics has been extensively studied during the past 40 years and is the basis of a multibillion dollar pesticide business. Fortuitously, plant enzymatic systems involved in metabolic and cometabolic processes can also detoxify pesticides. Microorganisms have similar processes and an intrinsic nature for rapid genetic adaptation to chemicals in the environment. The detoxification and degradation potential of individual microorganisms are being exploited for remediation of soil and water contaminated with pollutants of diverse chemical nature. One particular strategy for remediation of contaminated soils and water is phytoremediation, a process by which plants and their associated microorganisms collectively degrade, detoxify, and/or remove pollutants from these matrices. Microbial genes that encode for pesticide detoxification–degradation pathways have become pivotal for transforming plants into herbicide resistant crops. Many detoxification mechanisms are common to both higher plants and prokaryotic organisms. However, unique mechanisms for xenobiotic transformation are continually being elucidated in microbes, and novel xenobiotics and natural products with new chemistries are also being discovered.

This American Chemical Society (ACS) Symposium Series volume book is based on a symposium that took place at the 218th National Meeting of the ACS in New Orleans, Louisiana, August 22–26, 1999. This book provides an accumulation of some of the most recent research on enzymes from plants and microorganisms that catalyze pesticide metabolism. The purpose of this symposium and book on plant and microbial enzymes involved in pesticide transformation was to bring together scientists from a variety of disciplines: biochemistry, microbiology, plant physiology, and toxicology to present, summarize, review, and update information on xenobiotic metabolism. Specific enzymes and processes included the following: hydrolytic enzymes, glutathione and other conjugation mechanisms, cytochrome P-450 oxidases, peroxidases, nitroaromatic transformations, reductive dehalogenation, and so on.

It is hoped that this book will serve as a valuable information source for researchers involved in studies on the biotransformation, metabolism, and fate of pesticides. It is particularly hoped that this book will motivate researchers to become involved in these developing areas of plant and microbial enzyme research. The answer to many current questions that will improve our knowledge and technology can be found in understanding enzyme mechanisms, specificity, stability, regulation, and expression. This knowledge will promote an understanding for the safe and economical use of pesticides in the biosphere.

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.pr001

Acknowledgements

We Thank the participants for their outstanding contributions to the symposium and to this ACS Symposium Series volume. We are grateful to the ACS Division of Agrochemicals for providing financial assistance, a forum for this symposium and encouragement for this project. We express our sincere thanks and appreciation to all the cosponsors who have provided financial assistance: Monsanto-Canada, Monsanto-USA, Novartis, University of Guelph, USDA–ARS, and the Weed Science Society of America. We are also indebted to the peer reviewers for their timely and critical, and cogent assessment of these chapters. The outstanding efforts, assistance, and patience of Kelly Dennis and Anne Wilson of the ACS Books Department are also deeply appreciated.

J. CHRISTOPHER HALL
Department of Environmental Biology
University of Guelph
Guelph, Ontario N1G 2W1, Canada

ROBERT E. HOAGLAND
Southern Weed Science Research Unit
Agricultural Research Service
U.S. Department of Agriculture
Stoneville, MS 38776

ROBERT M. ZABLOTOWICZ
Southern Weed Science Research Unit
Agricultural Research Service
U.S. Department of Agriculture
Stoneville, MS 38776

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.pr001

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch001

Chapter 1

Pesticide Metabolism in Plants and Microorganisms: An Overview

R. E. Hoagland[1], R. M. Zablotowicz[1], and J. C. Hall[2]

[1]Southern Weed Science Research Unit, Agricultural Research Service, U.S. Department of Agriculture, Stoneville, MS 38776
[2]University of Guelph, Department of Environmental Biology, Guelph, Ontario N1G 2W1, Canada

Understanding pesticide metabolism in plants and microorganisms is a key component for the development, the safe and efficient utilization of these compounds, and for bioremediation of these chemicals in contaminated soil and water. Selective metabolism of pesticides in non-target species (e.g., crop plants) and sensitivity in target species (e.g. weeds, insects and pathogen pests), is the basis of chemical pest control. Pesticide biotransformations may occur via metabolism or co-metabolism. Metabolism of a given pesticide in plants and microorganisms is generally a multi-step process. Individual components of such degradation/detoxification pathways include: oxidation, reduction, hydrolysis and conjugation. Pathway diversity depends on the chemical structure of the xenobiotic compound, the organism, environmental conditions, and metabolic factors regulating expression of these biochemical pathways. Knowledge of these enzymatic processes, especially concepts related to mechanism of action, resistance, selectivity, tolerance, and environmental fate has advanced pesticide science. One example is the development of herbicide tolerant crops. Advances in pesticide metabolism have also been facilitated by use of improved analytical techniques, molecular biological approaches, and immunological tools.

The inception of synthetic organic compounds for control of insect, microbial, and weed pests began over sixty years ago. Pesticides have transformed agricultural production to provide the quality and quantity of food and fiber necessary for an exponentially increasing world population. Since that time, millions of tons of

In Pesticide Biotransformation in Plants and Microorganisms; Hall, J., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2000.

synthetic xenobiotic compounds have been used as pesticides, and the bulk of these compounds applied to the environment have been degraded via plant or microbial enzymes. Although most of these compounds have been developed, tested, and found safe to use according to label specifications, there is still some concern about the environmental risks associated with their use. Some of the older compounds initially introduced, e.g., DDT, [2,2-bis(4-chlorophenyl)-1,1,1-trichloroethane], aldrin [1,2,3, 4,10,10,hexachloro-1,4,4a,5,8,8a-hexahydro-1,4:5,8dimethanonaphthalene], chlordane [1,2,4,5,6,7,8,8-octachlor-2,3,3a,4,7,7a-hexahydro-4,7-methanodiane], etc., were highly persistent and bioaccumulated in food chains. However in most countries, their use has been curtailed, upon learning of their environmental hazards. Generally, pesticides are applied to soil, plants growing on soil, or aquatic areas. Off-target movement of pesticides can occur via leaching, runoff, volatilization, drift, excessive application, and accidental spills.

It is highly probable that chemical technology will remain the core strategy for controlling pests in crop and non-crop land in the future. Increasing concern about environmental impact of pesticides has resulted in a governmental regulatory climate requiring more extensive knowledge of environmental fate, toxicology, and persistence of pesticides, as well as their metabolite residue levels in foods. These factors necessitate further study of degradation pathways, enzyme activities, enzyme regulation and expression, and genetics; especially as new chemicals are developed as pesticides.

Most pesticides are subject to extensive degradation in plants and the environment. Some degradation is due to chemical and physical transformations such as photolysis, autolysis, oxidation, reduction, rearrangements, and inactivation due to binding processes (i.e., to soils and macromolecules). However, enzymatic transformation is by far the major route of detoxification. This book encompasses the enzymatic transformations of a wide variety of pesticides, and presents the mechanisms, biochemistry, genetics and regulation of these processes in plants and microorganisms. This overview focuses on the broad aspects of pesticide metabolism in plants and microorganisms, and examines the importance of these biochemical pathways for pesticide development and environmental stewardship. New methodologies that are broadening our knowledge of pesticide metabolism are discussed.

Enzymatic Basis of Pesticide Transformation

Many transformations of pesticides in plants and microorganisms occur via a process called co-metabolism. Co-metabolism can be defined as the biotransformation of an organic compound that is not used as an energy source, or as a constitutive element of the organism (*1*). Initial co-metabolic transformations may render pesticides less toxic to target and non-target organisms, and also may enhance pesticide vulnerability to

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch001

other biological, chemical, or physical degradative transformations. Enzymes involved in initial co-metabolic transformation include: hydrolytic enzymes (esterases, amidases, nitrilases, etc.), transferases (glutathione S-transferase, glucosyl transferases, etc.), oxidases (cytochrome P-450s, peroxidases, etc.), and reductases (nitroreductases, reductive dehalogenases, etc.). For complete degradation of a pesticide, several metabolic and or co-metabolic processes are usually required (*2*,*3*).

Many of the enzymes and detoxification/metabolic pathways of pesticides and xenobiotics are similar in plants and microorganisms. However, several fundamental differences exist among microorganisms and plants that affect xenobiotic metabolism (Table I).

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch001

Table I. Fundamental Differences Between Plant and Microbial Metabolism

Characteristic	*Microorganisms*	*Plants*
Nutrition	Typically heterotrophs; some chemoautrophs /photoautotrophs	Typically photoautotrophs
Aromatic ring cleavage	Numerous mechanisms for aerobic/anaerobic ring cleavage	Aromatic metabolites prone to conjugation / lignification
Terminal electron acceptors	Various: O_2, NO_3^-, NO_2^-, Fe^{+++}, Mn^{++}, SO_4^{--}	Limited to O_2
Metabolic sites	Degradative enzymes in periplasmic space, cytoplasm, or excreted extracellularly	Compartmentation of detoxification processes in vacuoles
Genetic regulation	Degradative genes typically arranged in operons	Transcriptionally regulated

There is a major distinction between plant and microbial metabolism with regard to carbon nutrition. Plants are typically photoautotrophs, acquiring energy from light, and carbon from CO_2 fixation. However, most microorganisms (except certain chemoautotrophs (nitrifying bacteria) and photoautotrophs (blue green algae) are dependent on organic compounds for their energy and carbonaceous cellular compounds. Cellular maintenance based on a heterotrophic existence, however, has provided adaptive processes for the development of metabolic pathways that utilize a wide range of organic compounds as energy sources. Soil microorganisms, such as various species of *Arthrobacter*, *Burkholderia*, *Pseudomonas*, *Sphingomonas*, can utilize a wide range of aliphatic, aromatic, and heterocyclic compounds. Basidiomycetes or “white rot fungi”, produce lignin degrading enzymes (lignin peroxidase and manganese peroxidase) that degrade natural polymers and certain recalcitrant xenobiotics (*4*).

Mineralization

Some microorganisms, especially bacteria and fungi, can completely metabolize a pesticide to CO_2, H_2O, inorganic nitrogen and other inorganic components via a process called mineralization (*1*). Mineralization is a multi-step process wherein initial catabolism occurs via hydrolytic or reductive reactions, and oxidation of aromatic ring components is also usually required. In individual bacterial species, mineralization of pesticides is not widespread, and this process rarely occurs in plants. Some selective examples of mineralization of herbicides, insecticides and fungicides by pure cultures of bacteria and fungi (*5-11*), are summarized in Table II. Historically, the phenoxy herbicides 2,4-D (2,4-dichlorophenoxyacetic acid) and 2,4,5-T (2,4,5-trichlorophenoxyacetic acid) are examples of pesticides mineralized by pure cultures of bacteria (e.g., *Alcaligines*, *Arthrobacter*, *Flavobacterium*, and *Pseudomonas*) (*12*). Some herbicides mineralized to CO_2 serve as a primary nitrogen source rather than a carbon source, e.g., atrazine [6-chloro-*N*-ethyl-*N'*-(1-methylethyl)-1,3,5-triazine-2,4-diamine] for *Pseudomonas* sp. ADP (*5*) and the bipyridilium

Table II. Selected Examples of Pesticide Mineralization by Pure Cultures of Bacteria and Fungi

Pesticide	*Organism*	*Citation*
Herbicides		
Atrazine	*Pseudomonas* sp. ADP	*5*
2,4,5-T	*Phanerochaete chrysosporium*	*6*
Dicamba (3,6-dichloro-2-methoxybenzoic acid)	*Pseudomonas* sp.; *Moraxella* sp.	*7*
Paraquat	*Lipomyces starkei*	*8*
Insecticides		
Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl *N*-methylcarbamate)	*Arthrobacter* sp.	*9*
Lindane (γ-hexachlorocyclo-hexane)	*Sphingomonas paucimobilis*	*10*
Fungicides		
Pentachlorophenol	*Sphingomonas* sp.	*11*

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch001

herbicide paraquat (1,1'-dimethyl-4,4'-bipyridinium ion) for the yeast, *Lipomyces starkeyi* (*8*).

An essential metabolic factor in the mineralization of most aromatic pesticides is ring cleavage. Many organic pesticides contain one or more aromatic rings with multiple and diverse substitutions (halide, amino, nitro, alkyl moieties, etc.) that present barriers to enzymatic ring cleavage. Aerobic ring cleavage by bacteria requires dihydroxylation, most often catalyzed by dioxygenases (*13*). There are two general classes of dioxygenases, one catalyzes *ortho* (intra-diol), the other *meta* (extradiol) fission of catechol, thereby forming *cis*-muconic acid or 2-hydroxymuconic acid, respectively. Hydroxylation of aromatic rings may also be catalyzed by cytochrome P-450 monooxygenases and mixed function monooxygenases, in which only one of the oxygen atoms is incorporated into the substrate molecule, and the other is incorporated into water (*14*).

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch001

In soil and plant rhizospheres, microbial communities contain some microbial species that complement the metabolic or co-metabolic capabilities of individual species, thereby facilitating complete xenobiotic mineralization. For example, the complete mineralization of parathion was facilitated by a consortium of two bacterial species, a ***Pseudomonas stutzeri*** and a ***Pseudomonas aeuriginosa*** (*15*). The *P. stutzeri* strain initiated hydrolysis, but was unable to mineralize *p*-nitrophenol. The *P. aeuriginosa* strain mineralized *p*-nitrophenol, which also provided growth factors for the *P. stutzeri* strain. Other bacterial consortia are involved in degradation of pesticides such as propachlor [2-chloro-*N*-(1-methylethyl)-*N*-phenylacetamide](*16*) and coumaphos [phosphorothioic acid *O*-(3-chloro-4-methyl-2-oxo-2H-1-benzopyran-7-yl) *O*,*O*,-diethyl ether] (*17*).

By comparison, oxidative metabolism of aromatic compounds in plants is usually catalyzed by cytochrome P-450 enzymes, peroxidases, or mixed function oxidases (see chapter by Mougin *et al.*, this volume). Hydroxylated metabolites generated from these processes are usually conjugated or incorporated into lignin (see chapters by Hall *et al.*, and Sandermann *et al.*). Dioxygenases occur in plants and have important functions in various biosynthetic pathways. However, their role in pesticide metabolism has not been critically examined. For example, *p*-hydroxyphenylpyruvate dioxygenase (HPPD), a key enzyme in tocopherol and plastoquinone synthesis, catalyzes the formation of homogentisic acid. Triketone herbicides, e.g., sulcotrione {2-[2-chloro-(4-methylsulfonyl)benzoyl] cyclohexane-1-3dione}, resemble the endogenous substrate structure and inhibit HPPD (*18*). Recently the genes for HPPD were cloned from *Arabidopsis* and over-expressed in the same plant species (*19*). Such transformed plants are resistant to the triketone herbicides, at concentrations 5- to 10-fold higher than the wild type can tolerate.

Many microorganisms are capable of aerobic metabolism under anoxic conditions by using alternate terminal electron acceptors in lieu of oxygen, e.g., NO_3^-, NO_2^-, and MnO_2. Anaerobic microorganisms may use NO_3^-, NO_2^-, SO_4^{2-}, CO_2 and other acceptors (*20*). Under aerobic conditions, the organic substrates are transformed to

CO_2, while under anaerobic respiration the product is either CO_2 or CH_4. Certain microorganisms possess anaerobic or facultative anaerobic metabolism, thus enabling pesticide biotransformation under anoxic conditions. This concept is examined in other chapters on reductive dehalogenation and halorespiration by rhizosphere microorganisms (Barkovski), and microbial nitroaromatic metabolism (Zablotowicz *et al.*).

Integration of Metabolic Pathways

In general, plants metabolize a pesticide by a series of multi-phase processes with a wide range of metabolic diversity among various species (*21*). The primary detoxification step usually involves hydrolysis or oxidation, producing functional groups that are subject to secondary enzymatic conjugation to endogenous compounds such as glutathione (GSH), sugars, or organic acids. Further processing of the conjugated derivatives can occur by formation of bound residues, additional conjugation reactions, compartmentation into vacuoles, and/or extracellular deposition.

A key component in the regulation of plant metabolism is the transport of metabolites such as GSH- or glucosyl-conjugates, from the cytoplasm by specific ATPase-conjugate pumps. For example, in plants, initial detoxification of a chloroacetamide herbicide via GSH conjugation takes place in the cytoplasm. Peptidase hydrolysis of the γ-glutamyl and glycine moieties occurs in the vacuole (*22*). By contrast, GSH conjugation in bacteria occurs either in the cytoplasm, or the periplasmic space (*23*), with γ-glutamyl transpeptidase activity found in the cytoplasm (*24*). Certain bacterial GSTs function in the basal metabolism of compounds such as dichloromethane, pentachlorophenol, and γ-hexachlorocyclo-hexane, thus these compounds can serve as nutritional sources (see chapter by Vuilleumier).

Certain enzymes involved in xenobiotic metabolism in plants and microorganisms may be constitutively expressed, whereas, many pesticide degrading/detoxifying enzymes are inducible in plants and microbes. Genetic regulation of plant enzyme expression differs from that in bacteria. In prokaryotic organisms, genes responsible for biotransformation pathways are typically organized in operons. In an operon, genes governing various enzymes in a pathway are often closely linked as a cluster in the genome. Thus, expression of all enzymes in a metablolic pathway can be induced or repressed by the same effector. This is not the case in eukaryotic systems such as plants and animals. In these cases, a single gene is regulated by its own specific promoter. In these systems, a myriad of proteins influence expression, i.e., specific cytoplasmic proteins (WD proteins) regulate expression affecting transcription, although they never enter the nucleus (*25*). Complete understanding of plant regulation of plant gene expression is not fully understood.

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch001

Metabolism and Pesticide Development

Site of Action

Enzymes are often the actual target sites of many pesticides, e.g., protox inhibitors (herbicides), ergosterol synthesis inhibitors (fungicides), and acetylcholinesterase (insecticides). Toxicity of a pesticide is usually due to the inhibition of a target enzyme, or in some cases toxicity is caused by over-expression or over-stimulation of the target enzyme. Unfortunately some pesticides also interfere with enzymes in non-target organisms, e.g., the insecticide carbaryl (1-naphthyl *N*-methylcarbamate), a potent acetylcholinesterase inhibitor, can inhibit esterases and amidases in plants (*26*) and microorganisms (*27, 28*).

Pesticide Selectivity

Pesticide selectivity is dependent on factors including absorption, translocation to active sites, and metabolism. Generally, the most important herbicide selectivity mechanism in plants (crops and weeds) is differential metabolism. This can occur via activation (transformation of the parent molecule to a biologically active metabolite) or via detoxification. Detoxification is a mechanism by which an individual organism can render itself tolerant or resistant to the toxicological nature of a xenobiotic. Specific examples of plant selectivity based upon detoxification, include ß-oxidation of phenoxybutyric acids (*29*), hydrolytic de-esterification of diclofop-methyl {(±)-2-[4-(dichlorophenoxy)phenoxy]propanoic acid} (*30*), and sulfoxidation of thiocarbamate herbicides (*31*). Plants have diverse pathways for herbicide metabolism that can be highly substrate specific, both within and among species. This enzymatic specificity has resulted in the discovery and utilization of numerous herbicides that are differentially metabolized in weed and crop species (*32*). The rate of metabolism of a pesticide by crops and weeds is also an important consideration related to selectivity.

Most pesticides have been developed through synthesis and screening programs, or by serendipitous discovery. Development of pesticides in the future will increasingly rely on rationally designed molecules that deliver biological activity to target species, with minimal effects on non-target species. A greater understanding of structure, function and regulation of detoxifying/activating enzymes will enable and promote rational design in pesticide development.

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch001

Managing Herbicide Resistance in Weeds

Understanding pesticide detoxification mechanisms in crops and weeds is pivotal to the development of herbicide resistant weed management programs. Ideally, crop rotation and alternating the usage of herbicides with different modes of action are implemented in weed management strategies to minimize the evolution of resistant weed biotypes. There are many weeds that have evolved resistance to one or more herbicides (*33*). Studies of weed resistance to herbicides have indicated several mechanisms for resistance (*19*, *34-39*) (Table III). The most common mechanism for resistance is modification of target sites, while increased metabolism is much less frequent (*40*). Resistance mechanisms to several herbicides, based upon development of mutations in target enzyme active sites, have been assessed, e.g., acetyl-CoA carboxylase (ACCase), and acetolactate synthase (ALS) inhibitors (*41*) photosystem II inhibition due to mutations in the D1 protein (*34*).

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch001

Table III. Mechanisms of Herbicide Resistance

Mechanism	*Herbicide - plant: specific mechanism*	*Citation*
Target site resistance	Triazines - numerous: mutation in D1 protein	*34*
Target site over-expression	Sulcotrione - *Arabidopsis*: HPPD	*19*
Metabolic detoxification	Atrazine - velvetleaf: GSH conjugation	*35*
	Simazine - rigid ryegrass: *N*-dealkylation	*36*
	Chlortoluron - rigid ryegrass: *N*-dealkylation	*37*
Reduced activation	Triallate (proherbicide) - wild oat (*Avena fatua*): altered oxidase	*38*
Multiple resistance	Many herbicides - rigid ryegrass: several mechanisms	*39*

Propanil [*N*-(3,4-dichlorophenyl)propionate] resistance in barnyardgrass (*Echinochloa crus-galli*) and junglerice (*Echinochloa colona*) has been attributed to increased metabolism via enhancement of aryl acylamidase activity (*42, 43*). A population of atrazine resistant velvetleaf (*Abutilon theophrasti*) plants developed resistance by acquiring a new GST isozyme specific for the triazines (*35*). Simultaneous resistance to both simazine (6-chloro-*N*-*N'*-diethyl-1,3,5-triazine-2,4-diamine) and chlortoluron [*N'*-(3-chloro-4-methylphenyl)-*N*-*N*-dimethylurea] developed in rigid ryegrass (*Lolium rigidum*) via enhanced *N*-dealkylation (*36, 37*). A specific biotype of rigid ryegrass VLR69 was subsequently found that exhibited multiple resistance to nine classes of herbicides, 21 years after exposure to five herbicide classes (*39*). In this case, resistance may be conferred by several mechanisms including, decreased target-site sensitivity and increased metabolism. In areas where resistant weed populations already exist, the use of herbicide combinations or herbicide synergists may be useful to suppress resistant weeds.

Herbicide Resistant Crops

Herbicides generally act by inhibiting a specific enzyme of a major plant biochemical or physiological process. Recent biotechnological advances have led to the production of transgenic crop plants with resistance to specific herbicides. Prior to the advent of this biotechnology, conventional breeding was used to selectively define the traits of a crop genotype. This relatively slow process has resulted in the development of only one commercial crop cultivar, metribuzin [4-amino-6-(1,1-dimethylethyl)-3-(methylthio)-1,2,4-triazin-5(4*H*)-one] resistant soybean [*Glycine max* (L.) Merr.] (*44*). Tissue culture techniques have been used to select cell lines tolerant to various herbicides including; 2,4-D, picloram (4-amino-3,5,6-trichloro-2-pyridinecarboxylic acid), paraquat, chlorsulfuron {2-chloro-N-[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]benzenesulfonamide}, and imazaquin {2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1*H*-imidazol-2-yl]-3-quinolinecarboxylic acid}(*44*). In many cases, herbicide tolerance derived via selection from tissue culture, is either unstable or non-inheritable.

Today, nearly all herbicide-resistant crops are being commercially developed for resistance using cloned genes and molecular transformation techniques. Transgenic crops expressing foreign genes for detoxification have been developed, thereby conferring resistance to several herbicides. Two similar genes, *bar* (*45*) and *pat* (*46, 47*) confer resistance to glufosinate [2-amino-4-(hydroxymethylphosphinyl)butanoic acid] via a bacterial acetyl transferase gene, while resistance is imparted to bromoxynil (3,5-dibromo-4-hydroxybenzonitrile) and phenmedipham {3-[(methoxy-carbonylamino]phenyl (3-methylphenyl)carbamate} via bacterial genes for nitrilase (*bxn*) (*48*) and carbamate hydrolase (*49*), respectively. Discussions on these mechanisms are found elsewhere in this volume (e.g., chapters by Feng; Shaner and Techle; and Hoagland and Zablotowicz).

Pesticide Safeners, Antagonists and Synergists

The selectivity and tolerance of a particular herbicide may be improved by the use of other non-pesticidal compounds called safeners (*50*). Although many compounds have been discovered that can protect plants from phytotoxic compounds, their mode of action is not always fully understood. Generally, such safeners act by elevating the

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch001

metabolism of the herbicide, or by altering herbicide translocation in the protected species. Many safeners may elicit a variety of effects in plants, depending on species. This multiplicity of biochemical effects, and the finding that many weeds are generally not safened, has lead to some controversy. The role of safeners on enhancing GST and cytochrome P-450's is well documented. New and improved safeners may be developed when compounds that stimulate other detoxification mechanisms are discovered, and the factors influencing enzyme expression and regulation are elucidated. The use of safeners to induce metabolic detoxification of herbicides is examined in detail by Ramsey *et al.* in this volume. Specific information on the effects of safeners on expression of plant GST's is addressed later by Hatzios.

Microorganisms capable of rapidly degrading pesticides may also be applied to seed or soil and serve as a biosafeners. This concept has been demonstrated using bacterial inoculants for the herbicides dicamba (*51*), and chlorsulfuron (*52*), and the fungicide pentachlorophenol (*53*).

Early in the development of synthetic pesticides, combinations of certain agrochemicals were found to enhance or antagonize the action of a given pesticide. It is also common for growers to apply mixtures of pesticides, thus other interactions were discovered. Hatzios and Penner (*54*) summarized over 250 examples of herbicide interactions with herbicides, fungicides, insecticides and other agrochemicals; selected examples (*55-64*) are summarized in Table IV.

The insecticide carbaryl and other organophosphorous insecticides can synergize propanil phytotoxicity via aryl acylamidase inhibition in rice (*Oryza sativa*) causing injury to the crop (*26*). Aryl acylamidase is responsible for the hydrolytic detoxification of propanil, which renders the rice tolerant to this compound. Other compounds have been discovered to act synergistically with propanil and may be useful to control propanil-resistant barnyardgrass (*65*, *66*).

Monooxygenases are important in pesticide detoxification by plants (Mougin *et al.*, this volume). There is abundant evidence demonstrating synergistic effects of cytochrome P-450 inhibitors with various herbicides. A commonly used cytochrome P-450 inhibitor, piperonyl butoxide (PBO), can also enhance herbicidal activity of atrazine and terbutryn (2-(tert-butylamino)-4-(ethylamino)-6-(methylthio-s-triazine) in corn (*67*). PBO enhanced the effectiveness of thiazopyr [methyl 2-(difluoromethyl)-5-(4,5-dihydro-2-thiazolyl)-4-(2-methylpropyl)-6-(trifluromethyl)-3-pyrdinecarboxylate] in barnyardgrass, grain sorghum (*Sorghum vulgare*), and redroot pigweed (*Amaranthus retroflexus*) (*68*) by inhibiting thiazopyr detoxification in these species.

Grass species that are moderately tolerant (wheat, *Triticum aestivum*) and moderately susceptible (barley, *Hordeum vulgare*) to fenoxaprop-ethyl (FE) {(±)-2-[4-[(6-chloro-2-benzoxazolyl)oxy]phenoxy]propanoic acid ethyl ester} have higher amounts of GSH than species that are highly susceptible to FE, e.g., wild oat, barnyardgrass, smooth crabgrass (*Digitaria ischaemum*), and yellow foxtail (*Setaria glauca*) (*69*). FE is rapidly hydrolyzed to fenoxaprop (F) in all these species. In wheat and barley, the phenyl group of F is rapidly displaced by GSH and/or cysteine,

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch001

Table IV. Examples of Herbicide/Pesticide Interactions in Plants

Herbicide	*Pesticide*	*Interaction*	*Plant species*	*Citation*
Alachlor	Atrazine (H)	Antagonism	Oat (*Avena sativa*)	*55*
Alachlor	Atrazine (H)	Synergism	Barnyardgrass	*56*
Atrazine	Fenaminosulf (F)	Antagonism	Cucumber (*Cucumis sativus*)	*57*
Atrazine	Diazinon (I)	Antagonism	Soybean	*58*
Diclofop-methyl	2,4-D (H)	Antagonism	Oat	*59*
EPTC	Carboxin (F)	Antagonism	Corn (*Zea mays*)	*60*
Picloram	Mecoprop (H)	Synergism	Bean (*Phaseolus vulgaris*)	*61*
Propanil	Carbofuran (I)	Synergism	Tomato (*Lycopersicon esculentum*)	*62*
Trifluralin	PCNB (F)	Synergism	Cotton (*Gossypium hirsutum*)	*63*
Trifluralin	Aldicarb (I)	Synergism	Cotton	*64*

Note: F= fungicide, H=herbicide, I=insecticide.
Source: Adapted from Hatzios and Penner (*54*).

Note: Alachlor = 2-chloro-*N*(2,6-diethylphenyl)-*N*-(methoxymethyl)acetamide; Aldicarb = 2-methyl-2-(methylthio)propionaldehyde *O*-(methylcarbamaryl)oxime; Carboxin = 5,6-dihydro-2-methyl-N-phenyl-1,4-oxathiin-3-carboxamide; Diazinon = phosphorothioic acid *O,O*-diethyl *O*-[6-methyl-2-(1-methylethyl)-4-pyrimidinyl] ester; EPTC = *S*-ethyl dipropyl carbamothioate; Fenaminosulf = sodium [4-(dimethylamine)phenyl] diazenesulfonate; Mecoprop = (±)-2-(4-chloro-2-methylphenoxy)propionic acid; PCNB = pentachloronitrobenzene; Trifluralin = 2,6-dinitro-*N,N*-dipropyl-4-(trifluoromethyl)benzenamine.

resulting in formation of a GSH or cysteine conjugate, and 4-hydroxyphenoxypropanoic acid (*69-73*), with only low conjugation of F in the susceptible species. The triazole, fenchlorazole-ethyl (FCE), acts as a safener against the phytotoxic action of FE in wheat and barley, and as a synergist for the herbicide in barnyardgrass, smooth crabgrass or yellow foxtail (*74*). However, FCE has no synergistic or safening effect on FE toxicity to wild oat. This phenomenon is explained as follows. In the presence or absence of FCE, FE is rapidly converted to the toxophore F in the susceptible species, with little subsequent metabolism.

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch001

Synergism in barnyardgrass and yellow foxtail results from enhanced de-esterification of FE to phytotoxic F as a result of FCE (*70, 75*). This does not happen in oat or wild oat since FCE is rapidly metabolized to water-soluble metabolites before it can mediate enhanced FE de-esterification. In wheat and barley, FCE stimulates both the de-esterification of FE to F, and its subsequent degradation and conjugation to non-phytotoxic products. This phenomenon makes FCE unique in that it is an effective safener for a herbicide on certain crops, but can act as a synergist for the same herbicide on important monocot weeds.

Environmental Effects on Pesticide Metabolism in Plants

Various environmental parameters effect the efficacy and metabolism of many pesticides. Global change (elevated CO_2, air pollution, ozone, increased UV irradiation, etc.) appears to be a phenomenon that should be evaluated with regard to plant metabolic activities on xenobiotic compounds. Certain plant detoxification enzymes and cofactors, e.g., GST and GSH are involved in defense mechanisms triggered by environmental stress (*76*). Since glutathione conjugation is a major pathway of xenobiotic detoxification/deactivation, the relationship between elevated activity of such enzymes and pesticide metabolism should be investigated. Specific oxidases, such as superoxide dismutase, are elevated in response to ozone and may also play a role in enzymatic pesticide detoxification. Higher UV irradiation may lead to photolysis, activation/inactivation of xenobiotics, and formation of free radicals. A recent study evaluated tolerance to the herbicide glyphosate in two weed species; redroot pigweed (a C-4 plant) and *Chenopodium album* (common lambsquarters, a C-3 plant) (*77*). Results indicated that tolerance to glyphosate [*N*-(phosphonomethyl)glycine] increased in the C-3 plant as CO_2 concentrations were elevated. Thus, mechanisms of plant tolerance and susceptibility to pesticides may be altered in a detrimental manner in such a changing global environment.

Pesticide Fate in the Environment

Understanding pesticide fate is important for developing chemicals that have little or no impact on non-target organisms, and for defining their optimal dose and application method. Although the environmental fate of many pesticides is affected by chemical and physical processes, such as sorption/desorption and sequestration, knowledge of biological transformation pathways is a prerequisite to understand what metabolites may be present in the environment. This is important since certain pesticide metabolites possess greater plant or mammalian toxicity compared to the parent compound. The possibility of long-term exposure and chronic effects of these

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch001

metabolites also necessitates a need for their identification and study (see chapter by Shocken).

It is important to understand the major pathways of xenobiotic degradation in aquatic and terrestrial environments, and to determine the major groups of microorganisms responsible for such biotransformations. This has been accomplished for many of the common pesticides, and in many cases the metabolic pathways and enzymes responsible for pesticide metabolism have been characterized. The genes encoding several pesticide-degrading enzymes have been cloned and sequenced, and their genetic regulation has been elucidated, e.g., atrazine (see chapter by Sadowsky and Wackett).

Accelerated Biodegradation

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch001

Continued use of some soil-applied pesticides has resulted in reduced efficacy due to metabolic adaptation of microbial populations, and subsequent enhanced biodegradation potential in the exposed soil. This phenomenon has been observed with several insecticides (e.g., aldicarb and diazinon), herbicides (e.g., 2,4-D, EPTC, butylate [*S*-ethyl bis(2-methylpropyl)carbamothioate], and vernolate (*S*-propyl dipropyl carbamothioate), and fungicides (benomyl and ipridione [3-(3,5-dichlorophenyl)-*N*-isopropyl-2,4-dioxoimidazolidine-1-carboxamide]) (*78*). A microbial population may also be cross-conditioned for accelerated degradation by exposure to related compounds, e.g., a soil repeatedly treated with the thiocarbamate EPTC was adapted for rapid EPTC degradation, as well as vernolate and butylate (*78*). These relatively rapid microbial adaptations severely limit the effective use of such pesticides in these situations. However, such induced selection pressure could provide new biotypes of microbial strains useful for remediation of sites contaminated with certain pesticides.

Transfer of genes for pesticide degradation among bacterial populations can occur via several mechanisms. Some of the these genes are readily exchanged among members of the soil microbial community, especially those encoded on plasmids. Specific examples of plasmid-borne degradation genes include the *Achromobacter* sp. carbofuran hydrolase that hydrolyzes EPTC (*79*), the *Klebsiella pneumoniae* nitrilase that hydrolyzes bromoxynil (*80*), and a series of genes in *Pseudomonas* sp. ADP encoding enzymes for atrazine degradation (see chapter by Sadowsky and Wackett). Degradative traits that are encoded on plasmids may be lost, especially in the absence of selection pressure. Other pesticide transformation genes are flanked by repeated DNA sequences that resemble insertion sequences or transposons. Specific examples of insertion sequences include, the 2,4,5-T degrading genes of *P. cepacia* (*81*), and the atrazine-degrading genes in *Pseudomonas* strain ADP (see Sadowsky and Wackett, chapter this volume). Transfer of genes via insertion sequences may facilitate evolution of novel degradation pathways that are more stable than plasmid encoded genes. Optimal management of pesticide use depends upon knowledge of the microbial pathways for degradation, and the potential for transfer of these degradative traits among members of the microbial community.

Bioremediation

The majority of pesticides are transformed to nontoxic metabolites via enzymatic processes. Pesticides applied at recommended doses have minimal impact on the environment due to uptake and metabolism by plants and microbes. However, in some cases, pesticide concentrations unacceptable to human, animal, or ecosystem health, occur via non-point (leaching, runoff, etc.), or point source contamination (accidental spills, misuse, etc.). In these cases, selection of bioremediation strategies based upon microbial and plant enzymatic processes may be appropriate for reclamation.

Bioremediation of pesticide-contaminated soils and water may be achieved *ex situ* (removal of the contaminated matrix and treatment in a bioreactor), or more desirably, via *in situ* remediation. Contaminated groundwater and other aqueous systems are suitable for treatment in a bioreactor system, especially when immobilized cells of degrading organisms are used. When a propachlor-degrading bacterial strain (*Pseudomonas* sp. GCH1), was used in such a bioreactor system, 98% of a propachlor sample (0.5 mM) was removed during a 3-h retention time (*82*).

Several strategies may be utilized for *in situ* bioremediation of pesticide-contaminated soils, e.g., bioaugmentation, biostimulation, and intrinsic remediation. Bioaugmentation involves inoculation of the contaminated matrix with a microorganism or a microbial consortium possessing the desired degradation characteristics. This approach may be useful when the microbial population capable of degrading the chemical is either lacking, or very low. A useful technique for enhancing the co-metabolic potential of indigenous microorganisms is biostimulation (*83*). In this approach, the contaminated matrix is amended with a limiting factor (typically a carbon or nitrogen source) that elicits either a generic or specific stimulation of microbial activity. One successful example for remediating soil contaminated with the herbicide dinoseb (2-*sec*-butyl-2,4-dinitrophenol) consists of amending soil with a carbon source (potato starch), and allowing the development of anaerobic conditions, which facilitates aromatic nitroreduction and development of other populations capable of anaerobic ring cleavage (*84*). Intrinisic remediation assumes that there is an inherent potential in native microorganisms to degrade a given compound, and that during a certain time period, their biodegradative potential will be expressed. Intrinsic remediation may use processes such as biosparging with oxygen, or tillage to provide a more suitable environment for degradation.

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch001

Phytoremediation

Phytoremediation is a relatively new technology using living plants for the removal and metabolism of contaminants from polluted soil and water (*85*, *86*). Plants can remove environmental contaminants due to their ability to extract water and solutes from the soil/water matrix. The root zone of plants is also an important ecological niche for accelerated biodegradation of contaminants, primarily due to the proliferation of microorganisms in the soil:root interface known as the rhizosphere. Although phytoremediation is an emerging technology, an understanding of the mechanisms of xenobiotic metabolism in plants and microorganisms, as well as the ecology of plant-microbial interactions for optimizing contaminant removal is rapidly developing (*87*).

A recent study indicated that use of a 2,4,6-trinitrotoluene (TNT) -cometabolizing *Pseudomonas* sp. (strain I4), together with an appropriate plant species (meadow brome, *Bromus erectus* Huds.) was effective in removing TNT from contaminated soil (*88*). However, planting contaminated soil with this species, or inoculation with strain I4 as individual treatments, did not reduce TNT content of soil. The inoculant strain co-metabolized TNT via aromatic nitroreduction, but was unable to completely degrade it. Despite this inability to completely metabolize TNT, nitroreduction sufficiently reduced TNT phytotoxicity to allow plant growth. Rhizosphere stimulation subsequently enhanced other degrading bacterial populations possessing the *ntdA* or *ntn*M genes specific for 2-nitrotoluene and 4-nitrotoluene degradation. Defining the optimized integrated system of appropriate plant species, microbial inoculants, and environmental constraints is crucial in establishing a viable phytoremediation system. Further technology is considering plant species genetically modified for enhanced phytoremediation, e.g., poplar trees for enhanced metabolism of trichloroethylene (*89*).

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch001

Technology and Methodology

Advances in pesticide science, particularly studies on mode/mechanism of action, metabolism, and metabolic regulation, cannot be accomplished efficiently without the development of technology and methodology. Several advances in technology as outlined below, have been made in the past few years that will aid pesticide scientists.

Molecular Biology Techniques

The use of molecular biology techniques by pesticide scientists has increased greatly in the past 5-10 years. The polymerase chain reaction (PCR) technique, restriction fragment length polymorphism (RFLP), random amplification polymorphic DNA (RAPD), gene cloning, and the use of gene libraries have facilitated the determination of pesticide resistance mechanisms, target sites of pesticides, and genetic differences between weed biotypes and species. Use of molecular biology techniques has the potential to advance the current concepts of biology and evolutionary aspects of enzymes related to plant and microbial degradation of pesticides. DNA chips and microarrays for use in functional genomic studies (*90*) are molecular biology tools useful in determining genetic differences between resistant and sensitive biotypes, and in identifying genes that are expressed in response to pesticide exposure.

PCR techniques have been adapted by environmental microbiologists for quantitative assessment of specific microbial populations via most-probable number-PCR (*91*), and competitive-PCR (*92*). These respective techniques have been used to enumerate pentachlorophenol-degrading *Sphingomonas* strains based upon the *pcp*C gene, and populations of a *Desulfitobacterium frappieri* strain during anaerobic degradation of chlorinated phenols. The role of microbial communities and consortia in xenobiotic degradation has been advanced by using ARDRA (amplified ribosomal DNA restriction analysis) coupled with either denaturing gel electrophoresis (DGGE), or temperature gradient gel electrophoresis (TGGE) (*93*). The application of DGGE to study changes in microbial populations affected by plant products that induce microbial degradation is discussed in a chapter by Crowley *et al.*, in this volume. Oligonucleotide microchips had utility for quantification of multiple microbial populations in environmental samples when the small rRNA subunit was used as the target probe (*94*). Studying gene expression in environmentally important bacteria *in situ* has been possible with the advent of reverse transcription-PCR (RT-PCR) (*95*). Specific messages are amplified inside the cell using RT-PCR, and are detected via hybridization with labeled probes that can be visualized microscopically. All these techniques will foster the study of microbial xenobiotic metabolism in the future.

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch001

Genomics

The study of gene structure and function (i.e., genomics) of plants will profoundly influence basic research in plant biology, physiology, and crop improvement during the next decade (*90*). Hieter and Boguski (*96*) refer to functional genomics as the "development and application of global (genome-wide or system-wide) experimental approaches to assess gene function using the information provided by structural genomics".

The complete genome sequence of *Arabidopsis thaliana* will be available soon, and significant progress is being made in sequencing of the rice and soybean genomes (*97*). Sequencing of other crop genomes is also in progress. Integrated use of genomic sequence information, specifically expressed sequence tags (ESTs), gene array tools, and collections of mutant transgenic plant lines, will promote the discovery of pesticide mechanism of action. For example, herbicide researchers will soon have access to the complete inventory of all possible herbicide targets. Through the evaluation of the consequences of gene "knock out" or partial silencing on plant viability, herbicide target sites will be more easily distinguished from many non-target sites. Thomas (*97*) states that "herbicide tolerant reporter lines may also be identified by screening large populations of "knock in" mutants. These reporters may be used to screen cascades for the early diagnosis of specific modes of action.

Two molecular approaches to sustain herbicide discovery have been described, i.e., target validation and target identification (*98*). With the first approach, *in vivo* methods for target validation may be achieved using antisense inhibition of putative target genes in transgenic plants. Antisense inhibition can be used to verify the potential effects a herbicidal compound may have on the target, and also gives some indication of the threshold levels required to obtain effective target enzyme inhibition. The second approach involves identifying unknown target sites using the T-DNA activation tagging technique (*99*). Plants develop herbicide resistance by several mechanisms including, enhanced metabolism, altered uptake, mutation of the target site, and amplification of the target enzyme due to gene amplification or increased expression of the respective RNA. In the latter case, over-expression of a gene results in increased levels of RNA, which can be used to identify the target site using the T-DNA activation tagging technique. This method exploits the fact that a gene of interest can be randomly enhanced. Enhancement of the gene of interest is confirmed using the phytotoxin (i.e., herbicide) to screen the altered plant cells for increased tolerance or resistance. This process is used to develop a positive cell line, which can be screened to identify the gene, which can then be cloned. Further details on the role of new genomic technologies for discovery of herbicides and identification of their target sites have been summarized (*97*, *98*, *100*, *101*).

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch001

Chemical Analytical Techniques

Effective extraction methodologies have been developed with capabilities beyond conventional sonication, shaking, and homogenizing. Microwave-assisted extraction (MAE) uses microwaves and solvents to extract compounds from various matrices. Supercritical fluid extraction (SFE) and pressurized liquid extraction (PLE, also known under the trade name of accelerated solvent extraction, ASE^{TM}), utilize high pressure and elevated temperatures to extract compounds from various matrices. SFE extracts an organic compound with a pure substance (commonly CO_2) above its critical temperature and pressure, resulting in a supercritical fluid. Recovery of a solute with supercritical fluid extraction depends on the solubility of the analyte in the supercritical fluid, and the diffusion of the analyte and the supercritical solvent through the sample matrix. ASE uses conventional liquid solvents at elevated temperatures and pressures to extract solid and semi-solid samples.

An important component of any tool used in metabolic studies is that it must not introduce artifacts or cause non-metabolic breakdown products. Artifacts or breakdown products can produce false leads when attempting to elucidate metabolic pathways. Thus these new technologies must be critically assessed prior to use. Generally, these extraction methods are time efficient, require less solvent, and may be more selective than traditional techniques. All of these extraction methods have a wide range of applications including selective removal of pesticides from soils, tissues, foods, and water.

Extraction and purification methods for enzymes and other proteins are also crucial in elucidating mechanisms of pesticide metabolism. Although enzyme activity can be studied *in vivo* or *in vitro*, conclusive proof of a pathway typically requires enzyme extraction, isolation, and purification. Selection of appropriate extraction and purification schemes is important because enzyme activity can be lost and some proteins are readily denatured. A variety of methods have been used to isolate and purify enzymes, including precipitation, molecular filtration, ion exchange chromatography, electrophoresis, etc. More recently, fast protein liquid chromatography (FPLC) has been useful in the isolation of individual isozymes of GSTs from atrazine-resistant and -susceptible velvetleaf biotypes (*35*).

Traditional analytical methodology and instrumentation, e.g., gas chromatography (GC); infrared (IR), ultraviolet (UV), and visible spectrometry; gas chromatography-mass spectroscopy (GC-MS); high performance liquid chromatography (HPLC); and thin layer chromatography (TLC) have contributed significantly to the knowledge of biochemical and enzymological processes. Vast improvements in analytical technology over the past decade have facilitated rapid identification, structural determination, and stereochemical assignment of pesticides and their metabolites. Detection sensitivity has improved from the microgram to picogram levels, allowing more complete assessment of metabolic intermediates and minor products. Increased analytical speed has improved the detection of transient intermediates that are important in understanding transformation mechanisms. Nuclear magnetic resonance (NMR) is a spectroscopic technique that uses proton magnetic resonance to interpret the structure, and identity of organic compounds. NMR provides information on the type and number of functional groups present, thus aiding molecule identification. By contrast, IR and UV spectrometry measure the unique absorbance patterns of molecules exposed to different wavelengths of energy. The absorbance pattern of an unknown compound can be compared or matched with known compounds to facilitate identification. Moreover, IR provides information about functional groups of a molecule.

Mass spectrometry (MS) is a powerful, versatile tool for identification and structural analysis of molecules based on identification of the mass of fragments derived from the original molecule. The development of HPLC-MS has been useful in pesticide metabolism studies, especially for direct analysis of polar and nonvolatile compounds. A new development, matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry, is used both as an ion source and an ion separating method. MALDI-TOF is a technique whereby ions are formed by an intense pulse of energy from a UV laser beam (*102*). MALDI-TOF is used to ionize large molecules such as proteins, thus providing structural information and possible identification. All of these spectrometric methods can provide valuable information on pesticides and their metabolites.

Antibody Technology

Pesticide analysis has been based on the use of conventional techniques (GC, GC/MS, and HPLC) as pointed out above. Although these techniques are sensitive and reproducible, they are time consuming, expensive and require sophisticated technical

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch001

expertise for operation and equipment maintenance. Enzyme-linked immunosorbent assay (ELISA) on the other hand, provides a simple, rapid, and inexpensive method for the detection and quantification of pesticides in soil, water, air, and tissues (*103*). This technique has found application for numerous pesticides (herbicides, insecticides, and fungicides) and utility in detecting *Bacillus thuringiensis* toxin (Table V).

Table V. Selected Examples of ELISA Assays for Common Pesticides

Pesticide	*Citation*
Atrazine	*104*
Bacillus thuringiensis toxin	*105*
Benomyl [methyl 1-(butylcarbamoyl)benzimidazol-2-ylcarbamate]	*106*
Chloroacetamide herbicides	*107*
2,4-D	*108*
Diuron [*N*'-(3,4-dichlorophenyl)-*N*-*N*-dimethylurea]	*109*
Fluroxypyr-meptyl (1-methylheptyl 4-amino-3,5-dichloro-6-fluoro-2-pyridyloxyacetate)	*110*
Glyphosate	*111*
Metalaxyl [N-(2,6-dimethylphenyl)-N-(methoxyacetyl)-DL-alanine methyl ester]	*112*
Metosulam [N-2,6-dichloro-3-methylphenyl)-5,7-dimethoxy-1,2,4-triazolo(1,5a)-pyrimidine-2-sulfonamide]	*113*
Picloram	*114*
Triclopyr [(3,5,6-trichloro-2-pyridinyl)oxy]acetic acid	*110*

Traditionally, ELISA assays have been developed using polyclonal (from animals) and monoclonal (from tissue cultures) antibodies. With recombinant DNA technology, antibodies can be produced by other expression systems including bacteria, yeasts, and plants. Antibody genes are cloned from animals that are hyperimmunized with target antigen. These genes may be altered as required, and introduced into expression systems that produce the final antibodies. These recombinant antibodies can be used in immunoassays for environmental residual analysis (*115*, *116*). Antibodies produced by bacteria reduces the use of animals in research, and also lowers production costs.

Transgenic plants are practical and flexible experimental systems for the expression of foreign proteins such as antibodies. They are also one of the most economical systems for large-scale production of recombinant proteins for industrial and pharmaceutical uses (*117*, *118*). The first antibodies expressed in plants (plantibodies) were immunoglobulin genes (*119*). Individual tobacco plants were transformed to express either the heavy chain or the light chain gene of the antibody. These were crossed to produce a progeny expressing both chains. Since then, advances in recombinant antibody technology have allowed the introduction of both heavy and light chain genes in one construct (*120*, *121*, *122*), and the expression of different antibody fragments in plants (single-chain variable fragments, fragment

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch001

antibodies, and VH chains) (*123*, *124*). Expression of antibodies in plants may also impart herbicide resistant to the host plants, thereby reducing crop damage. Pesticide scientists seldom use antibody technology, yet the technology has broad applications.

Cytological Techniques

Modern cell biology techniques have been under-utilized methodologies by pesticide scientists, although these methods have excellent potential to provide new insights on cellular mechanisms of pesticide localization, sites of action, etc. This is particularly true considering the revolutionary developments in areas such as microscopy. Scanning electron microscopes equipped with cryostages allow preparation of samples with the surface waxes left intact, allowing the determination of effects of adjuvants on wax structure. The same technique, coupled with cathodoluminescence, can be used to detect the dispersion of a pesticide on the leaf surface. The laser-confocal microscope allows investigation of the subcellular distribution of herbicides or herbicide analogues in real time, in living specimens (*125*). This technique may revolutionize a field that now depends on autoradiography. Antibodies for use in immunofluorescence and immunogold techniques have advanced current concepts of the subcellular sites of pesticide action, cytological changes occuring in resistant biotypes, and those induced by pesticides (*126*). To date, these techniques have been utilized in only a few laboratories, but have produced promising results.

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch001

Structure-Activity Relationships and Pesticide Design

Quantitative-structure activity relationships (QSAR) may aid in the design and synthesis of more effective pesticides with optimal selectivity and persistence. QSAR has been used to assess mutagenicity, enzyme inhibitor potency, phamacological activity, and toxicology of various compounds. Computer-aided molecular design has resulted in enhanced use of QSAR in the design of biologically active compounds. Gene cloning and sequencing of enzymes, coupled with X-ray crystallography and NMR, have increased the understanding of enzyme structure, especially with regard to receptor sites (*127*). Specific enzymes have been characterized using QSAR, i.e., cholinesterase, carbonic anhydrase, chymotrypsin and alcohol dehydrogenase (*127*). QSAR has been used to assess the efficacy of derivatives of ergosterol-inhibiting fungicides (*128*), protox-inhibiting herbicides (*129*), and octopaminergic insecticides/miticides (*130*). However, thus far no novel agrochemical has been developed using QSAR.

Concluding Remarks

Since the beginning of pesticide development in the 1940s, tremendous progress has been made in understanding plant and microbial metabolism of these compounds.

This knowledge has provided a continuous and renewable wealth of information that has advanced the agrochemical industry and transformed modern agriculture. This overview has examined various components of pesticide biotransformations, summarized some new techniques that will revolutionize pesticide science, and outlined how this knowledge is being used in pest control and for the benefit of the environment.

Biotechnology methods have dramatically changed pest control within the past ten years, and exciting new directions are continually being explored. The new technologies that have been reviewed here; molecular biology, genomics, and immunology will be paramount in future biotechnological approaches. Computer simulation studies will continue to enhance our knowledge of enzymes, especially in the areas of structure elucidation, identification of catalytic intermediates, and rational design for enzyme engineering. Plant and microbial genomics, and DNA-microarray techniques will provide valuable tools for evaluating compounds being developed by combinatorial chemistry. Nevertheless, these advances must still rely on further developments in whole plant and microbial physiology. Although these technologies are adding value to methods of agricultural production, the net effect on profitability to the farmer and sustainability of the agroecosystem also needs to be considered.

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch001

Literature Cited

1. Alexander, M.A. *Biodegradation and Bioremediation*; Academic Press: San Diego, 1994; pp 177-193.
2. Hatzios, K.K. In *Environmental Chemistry of Herbicides, Vol. II*; Grover, R.; Cessna, A.J., Eds.; CRC Press: Boca Raton, FL, 1991; pp 141-185.
3. Shimabukuro, R.H. In *Weed Physiology: Herbicide Physiology, Vol. II*; Duke, S.O., Ed.; CRC Press: Boca Raton, Fl, 1985; pp 215-240.
4. Bumpus, J.A. In *Soil Biochemitry*; Bollag, J.-M; Stotzky, G., Eds.; Marcel Decker: New York, NY, 1993; Vol. 8, pp 65-100.
5. Mandelbaum, R.T.; Allan, D.T.; Wackett, L.P. *Appl. Environ. Microbiol.* **1995,** *61,* 1451-1457.
6. Ryan, T.P.; Bumpus, T.A. *Appl. Microbiol. Biotechnol.* **1989,** *31,*1387-1398.
7. Krueger, J.P.; Butz, R.G.; Atallah, Y.H.; Cork, D.J. *J. Agric. Food Chem.* **1989,** *37,*534-538.
8. Carr, R, J. G.; Bilton, R.F.; Atkinson, T. *Appl. Environ. Microbiol.* **1985,** *49,* 1290-1294.
9. Ramanand, K.; Sharmilla, M.; Sethunathan, N. *Appl. Environ. Microbiol.* **1988,** *54,* 2129-2133.
10. Nagasawa, S.; Kikuchi, R.; Nagat, Y.; Takagi, M.; Matsuo, M. *Chemosphere* **1993,** *26,* 1719-1728.
11. Leung, K.T.; Cassidy, M.B.; Shaw, K.W.; Lee, H.; Trevors, J.T.; Lohmeirer-Vogel, E.M.; Vogel, H.J. *World J. Microbiol. Biotechnol.* **1997,** *13,* 305-313.
12. Häggbloom, M.M. *FEMS Microbiol. Rev.* **1992,** *103,* 29-72.
13. Mason J.R.; Cammuck, R. *Annu. Rev. Microbiol.* **1992,** *46,* 277-305.

14. Mason J.R. *Intl. Ind. Biorechnol.* **1988,** *8, 19-24.*
15. Daughton, C.G.; Hseih, D.P.H. *Appl. Environ. Microbiol.* **1977,** *34,* 175-184.
16. Villareal, D. T., Turco, R. F.; Konopka, A. *Appl. Environ. Microbiol.* **1991,** *57,* 2135-2140.
17. Shelton, D.R.; Hapemann-Somich, C. J.; In *In-Situ and on Site Bioreclamation*; Hinchee, R.E.; Olfenbuttel, R.F. Eds.; Butterworth-Heinemann: New York, NY,1991; pp 313-323.
18. Secor, J. *Plant Physiol.* **1994,** *106,* 1429-1433.
19. Bartley, G.E.; Maxwell, C.A.; Wittenbach, V.A.; Scolink, P.O. *Plant Physiol.* **1997,** *113,* 1465.
20. Ehrlich, H.L. In *Soil Biochemistry*; Bollag, J.-M.; Stotzky, G. Eds.; Marcel Dekker Inc.: New York, NY, 1995; Vol. 8, pp 219-247.
21. Kreuz, K.; Martinoia, E. In *Pesticide Chemistry and* Bioscience; Brooks, G.T.; Roberts, T.R. Eds.; Royal Society of Chemistry: Cambridge, United Kingdom 1999; pp 279-297.
22. Coleman, J.O.D.; Randall, R.; Blake-Kaff, M.M.A. *Plant Cell Environ.* **1997,** *20,* 449-460.
23. Allocati, N.; Cellini, L.; Aceto, A.; Iezzi, T.; Angelucci, S.; Robuffo, I.; Dillio, C. *FEBS Lett.* **1994,** *354,* 191-194.
24. Zablotowicz, R.M.; Hoagland, R.E.; Locke, M.A.; Hickey, W.J. *Appl. Environ. Microbiol.* **1995,** *61,* 1054-1060.
25. Ma, H. *Plant Mol. Biol.* **1994,** *26,* 1611-1636.
26. Frear, D.S.; Still, G.G. *Phytochemistry* **1968,** *7,* 913-920.
27. Blake, J.; Kaufman, D.D. *Soil Biol. Biochem.* **1973,** *5,* 297-30.
28. Hoagland, R.E.; Zablotowicz, R.M. *Pestic. Biochem. Physiol.* **1995,** *52,* 190-200.
29. Wain, R.L.; Smith, M.S. In *Herbicides: Physiology, Biochemistry, Ecology,* Vol 1; Audus, L.J., Ed.; Academic Press: London, England, 1976; pp 279-302.
30. Shimabukuro, R.H.; Walsh, W.C.; Hoerauf, R.A. *J. Agric. Food Chem.* **1979,** *27,* 615-623.
31. Carringer, R.D.; Rieck, C.E.; Bush, L.P. *Weed Sci.* **1978,** *26,* 157-160.
32. Brown, H.M.; Fuesler, T.P.; Ray, T.P.; Strachan, S.D. In *Pesticide Chemistry: Advances in International Research, Development, and Legislation*; Frehse, H.M. Ed.; Proc 7th Intl. Congr. Pestic. Chem. (IUPAC), VCH Verlagsgsellschaft: Weinhem, Germany, 1991; pp 257-266.
33. Heap, I.M. *Pestic. Sci.* **1997,** *51,* 235-243.
34. Trebst, A. In *Herbicide Resistance in Weeds and Crops*; Caseley, J.C.; Cussans, G.W.; Atkin, R.K., Eds.; Butterworth-Heinemann: Oxford, UK, 1991; pp 145-164.
35. Anderson, M.P.; Gronwald, J.W. *Plant Physiol.* **1991,** *96,* 104-109.
36. Burnet, M.W.M.; Loveys, B.R.; Holtum, J.A.M.; Powles, S.B. *Pestic. Biochem. Physiol.* **1993,** *46,* 207-218.
37. Burnet, M.W.M.; Loveys, B.R.; Holtum, J.A.M.; Powles, S.B. *Planta* **1993,** *190,* 182-189.

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch001

38. Kern, A.J.; Peterson, D.M.; Miller, E.K.; Colliver, C.C.; Dyer, W.E. *Pestic. Biochem. Physiol.* **1996,** *56,* 163-170.
39. Preston, C.; Tardiff, F.J.; Christopher, J.T.; Powles, S.B. *Pestic. Biochem. Physiol.* **1996,** *54,* 123-130.
40. Saari, L.L. In *Pesticide Chemistry and Bioscience*; Brooks, G.T.; Roberts, T.R., Eds.; Royal Society of Chemistry: Cambridge, United Kingdom, 1999; pp 207-220.
41. Devine, M.D.; Eberlein, C.V. In *Herbicide Activity: Toxicology, Biochemistry and Molecular Biology*; Roe, R.M.; Burton, J.D.; Kuhr, R.J., Eds.; IOS Press: Amsterdam, 1997, pp 159-185.
42. Carey, V.F.; Hoagland, R.E.; Talbert, R.E. *Pestic Sci.* **1997,** *49,* 333-338.
43. Leah, J.M.; Caseley, J.C.; Riches, C.R.; Valverde, B. *Pestic Sci.* **1994,** *42,* 281-289.
44. Marshall, G. In *Herbicide Resistance in Weeds and Crops*; Caseley, J.C.; Cussans, G.W.; Atkin, R.K., Eds.; Butterworth-Heinemann Ltd.: Oxford, UK, 1991; pp 331-341.
45. DeBlock, M.; Botterman, J.; Vanderwiele, M.; Dockz, J.; Thoen, C.; Gossele, V.; Movra, N.R.; Thompson, C.; van Montague, J.; Leemans, J. *EMBO J.* **1987,** *6,* 2513-2520.
46. Broer, I ; Arnold, W.; Wohlleben, W.; Pühler, A. In *Proc. Braunschweig Symp. Applied Plant Molecular Biol.*; Galling: Braunschweig, Germany, 1989; p 240.
47. Wohlleben, W.; Arnold, W.; Broer, I.; Hillemann, D.; Strauch, E.; Pühler, A. *Gene* **1988**, *70*, 25-37.
48. Stalker, D.M.; McBride, K. E.; Malyj, L. B. *Science* **1988,** *242,* 419-423.
49. Streber, W. R.; Kutschka, U.; Thomas, F.; Pohlenz, H. D. *Plant Mol. Biol.* **1994,** *25,* 977-987.
50. Hatzios, K.K.; Hoagland, R.E. *Crop Safeners for Herbicides*, Academic Press: San Diego, CA, 1989; 400 pp.
51. Kruger, J.P.; Butz, R.G. *J. Agric. Food Chem.* **1991,** *39,* 1000-1003.
52. Gavrilkina, M.V.; Filipshanova, L.I.; Zimenko, T.G. *Vestsi. Akad. Navuk. Belarusi, Ser. Biyal. Navuk.* **1995,** *3,*55-58.
53. Pfender, W.F. *J. Environ. Qual.* **1996,** *25,* 1256-1260.
54. Hatzios, K.K.; Penner, D. *Rev. Weed Sci.* **1985,** *1,* 1-63.
55. Buckholtz, D.L.; Lavy, T.L. *J. Agric. Food Chem.* **1978,** *26,* 520-524.
56. Akobundu, I.I.; Duke, W.B.; Sweet, R.D.; Minotti, P.L. *Weed Sci.* **1975,** *23,* 43-48.
57. Nash, R.G.; Harris, W.G. *J. Environ. Qual.* **1973,** *2,* 493-497.
58. Penner, D. *Agron. J.* **1974,** *66,* 107-109.
59. Hall, J.C.; Edgington, V.; Switzer, C.M. *Weed Sci.* **1982,** *30,* 672-676.
60. Leavitt, J.R.C.; Penner, D. *Weed Res.* **1978,** *18,* 281-286.
61. Hamill, A.S.; Smith, L.W.; Switzer, C.M. *Weed Sci.* **1972,** *20,* 226-229.
62. Chang, F.Y.; Smith, L.W.; Stephenson, G.R. *J. Agric. Food Chem.* **1971,** *19,* 1183-1186.
63. Pinckard, J.A.; Standifer, L.C. *Plant Dis. Rep.* **1966,** *50,* 172-174.
64. Bolling, J.C.; Hacskaylo, J. *J. Econ. Entomol.* **1966,** *59,* 1026-1027.

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch001

65. Norsworthy, J.K.; Talbert, R.E.; Hoagland, R.E. *Weed Sci.* **1999,** *47,* 13-19.
66. Hoagland, R.E.; Norsworthy, J.K.; Talbert, R.E. *Pestic. Sci.* **1999,** *55,* 571-573.
67. Varsano, R.; Rabinowitch, H.D.; Rubin, B. *Pestic. Biochem. Physiol.* **1992,** *42,* 174-182.
68. Rao, S.R.; Feng, P.C.C.; Schafer, D.E. *Pestic Sci.* **1995,** *45,* 209-213.
69. Tal, A.; Hall, J.C.; Stephenson, G.R. *Weed Res.* **1995**, *35,* 133-139.
70. Romano, M.L.; Tal, A.; Yaacoby, T.; Stephenson, G.R.; Hall, J.C. *Brighton Crop Protection Conference – Weeds* **1991,** *3,* 1087-1094.
71. Romano, M.L.; Stephenson, G.R.; Tal, A.; Hall, J.C. *Pestic. Biochem. Physiol.* **1993**, *46,* 181-189.
72. Yaacoby, T.; Hall, J.C.; Stephenson, G.R. *Pestic. Biochem. Physiol.* **1991,** *41,* 296-304.
73. Tal, A; Romano, M.L.; Stephenson, G.R.; Schwann, A.L.; Hall, J.C. *Pestic. Biochem. Physiol.* **1993**, *46,* 190-199.
74. Hall, J.C.; Stephenson, G.R. *Brighton Crop Prot. Conf.-Weeds* **1995,** *1,* 261-268.
75. Stephenson, G.R.; Tal, A.; Vincent, N.A.; Hall, J.C. *Weed Res.* **1993,** *7,* 163-168.
76. Field, J.A.; Thurman, E.M. *Environ. Sci. Technol.* **1996,** *30,* 1413-1418.
77. Ziska, L.H.; Teasdale, J.R.; Bunce, J.A.; *Weed Sci.* **1999,** *47,* 608-615.
78. Felsot, A.S.; Shelton, D.R. In *Sorption and Degradation of Pesticides and Organic Chemicals in Soil*; Linn, D.M.; Carski, T.H.; Brusseau, M.L.; Chang, F.-H., Eds.; SSSA Special Publication No. 32, Soil Science Society of America: Madison, WI, 1993; pp 227-252.
79. Tomasek, P.H.; Karns, J.S. *J. Bacteriol.* **1989,** *171,* 4038-4044.
80. Stalker, D.M.; McBride, K.E. *J. Bacteriol.* **1987,** *109,* 955-960.
81. Tomasek, P.H.; Frantz, B.; Sangodkar, U.M.X.; Haugland, R.A.; Chakrabartty, A.M. *Gene* **1989,** *76,* 227-238.
82. Martin, M.; Mengs, G.; Plaza, E.; Garbi, C.; Sánchez, M.; Gibello, A.; Gutierrez, F.; Ferrer, E. *Appl. Environ. Microbiol.* **2000,** *66,* 1190-1194.
83. Zablotowicz, R.M.; Hoagland, R.E.; Locke, M.A. In *Pesticide Remediation in Soils and Water*; Kearney, P.C.; Roberts, T., Eds.; Wiley and Sons: Chichester, England, 1998; pp 217-250.
84. Kaake, R.H.; Roberts, D.J.; Stevens, T.O.; Crawford, R.L.; Crawford, D.L. *Appl. Environ. Microbiol.* **1992,** *58,* 1683-1689.
85. Cunningham, S.D.; Shann, J.R.; Crowley, D.E.; Anderson, T.A. In *Phytoremediation of Soil and Water Contaminants*; Kruger, E.L.; Anderson, T.A.; Coats, J.R., Eds.; Amer. Chem. Soc. Symp. Ser. 664, Amer. Chem. Soc.: Washington DC, 1997; pp 2-17.
86. Zablotowicz, R.M.; Hoagland, R.E. In *Microbial Biotechnology for Sustainable Development and Productivity*; Rajak, R.C., Ed.; Scientific Publishers: Jodhpur, India, 1999; pp 343-360.
87. Crowley, D.E.; Alvey, S.; Gilbert, E.S. In *Phytoremediation of Soil and Water Contaminants*; Kruger, E.L.; Anderson, T.A.; Coats, J.R., Eds.; Amer. Chem. Soc. Symp. Ser. 664, Amer. Chem. Soc.: Washington DC, 1997; pp 20-36.

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch001

88. Sisciliana, S.D.; Greer, C.W. *J. Environ. Qual.* **2000,** *29,* 311-316.
89. Newman, L.A.; Doty, S.L.; Gery, K.L.; Heilman, P.E.; Muiznieks, I.; Shang, Q.T.; Siemieniec, S.T.; Strand, S.E.; Wang, X.; Wilson, A.M.; Gordon, M.P. *J. Soil Contam.* **1998,** *7,* 531-542.
90. Somerville, C.; Somerville, S. *Science* **1999,** *285,* 380-382.
91. Leung, K.T.; Watt, A.; Lee, H.; Trevors, J.T. *J. Microbiol. Methods* **1997,** *31,* 59-66.
92. Beaudet, R.M.-J.; Levesque, J.; Villemur, R.; Lanthier, M.; Chénier, M.; Lépine, F.; Bisallion, J.-G. *Appl. Microbiol. Biotechnol.* **1998,** *50,* 135-141.
93. Muyzer, G.; Smalla, K. *Antonie van Leeuuwenhook* **1998,** *73,* 127-141.
94. Guschin, D.Y.; Mobararry, B.K.; Proudnikov, D.; Stahl, D.A.; Rittman, B.E.; Mirzabekov, A.D. *Appl. Environ. Microbiol.* **1997,** *63,* 2397-2402.
95. Hodgson, R.E.; Dustman, W.A.; Garg, R.P.; Moran, M.A. *Appl. Environ. Microbiol.* **1995,** *61,* 4074-4082.
96. Heiter, P.; Boguski, M. *Science* **1997,** *278,* 601-602.
97. Thomas, P.G. *Brighton Crop Prot. Conf.-Weeds* **1999,** *2,* 481-490.
98. Hőfgen, R.; Freitag, J.; Maimann, S. *Brighton Crop Prot. Conf.-Weeds* **1999,** *2,* 501-508.
99. Fritze, K.; Walden, R. In *Methods in Molecular Biology*; Gartland, K.M.A.; Davey, M.R., Eds.; Humanna Press Inc.: Totowa, NJ, 1995; Vol. 44, pp 281-294.
100. Berg, D., Tietjen, K., Wollweber, D., Hain, R. *Brighton Crop Prot. Conf.-Weeds* **1999,** *2,* 491-500.
101. McDermott, J.; Meurer, G.; Waters, B.; Wanggui, Y-S.; Pan, W.; Li, X.; Silva, C.; Brian, P.; Davies, J. *Brighton Crop Prot. Conf.-Weeds* **1999,** *2,* 509-518.
102. Pitt, A.R.. In *Protein Labfax*; Price, N.C., Ed.; Bios Scientific Publishers: Oxford, England, 1996; pp 174-186.
103. Hall, J.C.; Deschamps, R.J.A.; McDermott, M.R. *Weed Technol.* **1990,** *4,* 226-234.
104. Bushway, R.J.; Perkins, B.; Savage, S.A.; Lekousi, S.J.; Ferguson, B.S. *Bull. Environ. Contam. Toxicol.* **1988,** *40,* 647-654.
105. Cheung, P.Y.K.; Hammock, B.D. In *Biotechnology for Crop Protection*; Hedin, P.A.; Menn, J.J.; Hollingworth, R.M.; Eds.; Amer. Chem. Soc. Symp. Ser. 379, Amer. Chem. Soc.: Washington DC, 1988; pp 359-372.
106. Newsome, W.H.; Collins, P.G. *J. Assoc. Off. Anal. Chem.* **1987,** *70,* 1025-1027.
107. Feng, P.C.C.; Horton, S.R.; Sharp, C.R. *J. Agric. Food Chem.* **1992,** *40,* 211-214.
108. Fleeker, J.R. *J. Assoc. Off. Anal. Chem.* **1987,** *70,* 874-878.
109. Lee, N.; Skermitt, J.H.; Thomas, M.; Korth, W.; Bowmer, K.H.; Larken, K.A.; Ferguson, B.S. *Environ. Contam. Toxicol.* **1995,** *55,* 479-486.
110. Johnson, B.D.; Hall, J.C. *J. Agric. Food Chem.* **1996,** *44,* 488-496.
111. Clegg, B.S.; Stephenson, G.R.; Hall, J.C. *J. Agric. Food Chem.* **1999,** *47,* 5031-5037.
112. Newsome, W.H. *J. Agric. Food Chem.* **1985,** *33,* 528-530.

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch001

113. Parnell, J.S.; Hall, J.C. *J. Agric. Food Chem.* **1998,** *46,* 152-156.
114. Hall, J.C.; Deschamps, R.J.A.; Kreig, K.K. *J. Agric. Food Chem.* **1989,** *37,* 981-984.
115. Yau, K.Y.F.; Tout, N.L.; Trevors, J.T.; Lee, H.; Hall., J.C. *J. Agric. Food Chem.* **1998,** *46,* 4457-4463.
116. Webb, S.R.; Lee, H.; Hall, J.C. *J. Agric. Food Chem.* **1997,** *45,* 535-541.
117. Whitelam. G.C.; Cockburn, W.; Owen, M.R.L. *Trans. Plant Biochem.* **1993,** *22,* 940-944.
118. Franken, E.; Teuschel, U.; Hain, R. *Curr. Opin. Biotechnol.* **1997**, *8,* 411-416.
119. Hiatt, A.; Cafferkey, R.; Bowdish, K. *Nature* **1989,** *342,* 76-78.
120. During, K.; Hippe, S.; Kreuzaler, F.; Schell, J. *Plant Mol. Biol.* **1990,** *15,* 81-293.
121. Verch, T.; Yusibov, V.; Koprowski, V. *J. Immunol. Methods* **1998,** *220,* 69-75.
122. McCormick, A.A.; Kumagai, M.H.; Hanley, K.; Turpen, T.H.; Hakim, I.; Grill, L.K.; Tuse, D.; Levy, S.; Levy, R. *Proc. Natl. Acad. Sci. USA* **1999,** *96,* 703-708.
123. Dall'Acqua, W.; Carter, P. *Curr. Opin. Structural Biol.* **1998,** *8,* 443-450.
124. Hudson, P.J. *Curr. Opin. Biotechnol.* **1998,** *9,* 395-402.
125. *Cell Biological Applications of Confocal Microscopy*; Matsumoto, B., Ed.; Meth. Cell Biol. 38; Academic Press Inc.: San Diego, CA, 1993; 380 pp.
126. Sherman, T.D.; Vaughn, K.C. *Weed. Sci.* **1991,** *39,* 514-520.
127. Hansch, C.; Gao, H.; Hoekman, D. In *Comparative QSAR*; Devillers, J., Ed.; Taylor and Francis: Washington, DC, 1998; pp 285-368.
128. Reynolds, C.H.; Shaber, S.H. In *Computer Aided Molecular Design: Applications in Agrochemicals, Materials, and Pharmaceuticals*; Reynolds, C.H.; Holloway, M.K.; Cox, H.K., Eds.; Amer. Chem. Soc. Symp. Ser. 589, Amer. Chem. Soc.: Washington, DC, 1995; pp 171-182.
129 Reddy, K.N.; Dayan, F.E.; Duke, S.O. In *Comparative QSAR*; Devillers, J. Ed.; Taylor and Francis: Washington, DC, 1998; pp 197-234.
130. Dekeyser, M.A.; Harrison, W.A.; McDonald, P.T.; Angle, G.W. Jr.; Ismail, S.M.M.; Downer, R.G.H. In *Computer Aided Molecular Design: Applications in Agrochemicals, Materials, and Pharmaceuticals*; Reynolds, C.H.; Holloway, M.K.; Cox, H.K., Eds.; Amer. Chem. Soc. Symp. Ser. 589, Amer. Chem. Soc.: Washington, DC, 1995; pp 183-196.

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch001

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch002

Chapter 2

In Vitro Methods in Metabolism and Environmental Fate Studies

Mark J. Schocken

**Uniroyal Chemical Company, Inc., World Headquarters,
Middlebury, CT 06798**

In vitro methods can be very helpful in supporting agrochemical metabolism and environmental fate studies. Metabolites can often be produced in sufficient quantities to allow complete structure elucidation by spectroscopy (^{1}H-, ^{13}C- and ^{19}F-NMR, MS, IR and UV) as well as to serve as analytical reference standards. *In vitro* methods can also provide important information relevant to elucidation of degradation pathways, including identification of pathway intermediates. Intermediates are often not detected when conducting traditional metabolism and environmental fate studies where emphasis is often on the identification of terminal metabolites. *In vitro* approaches can also have predictive value that can provide great insight into likely metabolites and metabolism pathways for new "pipeline" products, prior to the conduct of extensive and often expensive metabolism studies using intact biological systems. Types of *in vitro* approaches to be discussed, with illustrative examples from the literature, include microbial, cytosolic and microsomal transformations, as well as a non-biological mimic of oxidative enzymes such as cytochrome P-450 monooxygenases.

In Pesticide Biotransformation in Plants and Microorganisms; Hall, J., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2000.

The use of *in vitro* methods can be very helpful in metabolism and environmental fate studies that are required for supporting an agrochemical registration. To first establish clarity, the dictionary defines *in vitro* as "outside the living body and in an artificial environment; literally, in glass" (*1*). *In vitro* methods may include microbial systems (whole cells, cell extracts or purified enzymes), plant systems (excised plant tissues, tissue culture [callus and cell suspension] and isolated enzymes), animal systems (organ perfusion, tissue slices, cell cultures, isolated hepatocytes, subcellular fractions such as microsomes and tissue homogenates such as liver).

Why use *in vitro* techniques? First, *in vitro* approaches can generate xenobiotic metabolites in sufficient quantities (i.e., milligrams) to allow identification by spectroscopic means (e.g., by ^{1}H- and ^{13}C-NMR, MS, IR and UV). Subsequently, depending on the difficulty in preparing reference standards by conventional chemical synthesis, metabolites can be synthesized by *in vitro* systems in larger quantities (i.e., milligrams to grams) to serve as metabolite analytical reference standards. Third, *in vitro* methods can often detect intermediates in a degradation pathway rather than focusing on terminal metabolites, thus providing greater mechanistic insight. Fourth, *in vitro* methods can predict metabolites in soil, animals and plants prior to conducting complicated and often quite expensive metabolism and environmental fate studies. This paper will survey a variety of *in vitro* approaches on a variety of compounds, taking illustrative examples from the literature. Attention will be focused on microbial areas, as that has been the author's particular research interest over the past 20 years.

The use of common soil microbes for the synthesis of potential metabolites of agrochemicals represents an important alternative to chemical synthesis (*2*). That is, the metabolism of xenobiotics (including agrochemicals and pharmaceuticals) by selected microbial species is often remarkably similar to that of mammals, birds, fish, soil and plants. This similarity in metabolite profiles can be explained in large part by the presence of the enzymes, cytochrome P-450 monooxygenases, in these lower organisms. These enzymes are capable of catalyzing aliphatic and aromatic hydroxylations as well as *N*-, *S*- and *O*-dealkylations on a very wide range of xenobiotic substrates. Because of metabolite profiles similar to those observed in biological systems required by the agrochemical industry (e.g., in rats, goats, poultry, fish, plants and soils), microbial cultures can be used to synthesize quantities of metabolites (e.g., milligrams) sufficient to obtain spectra for identification purposes. Subsequently, these metabolites can be generated in larger amounts through larger-scale fermentations to serve as reference standards in support of agrochemical metabolism and environmental fate studies.

In addition to providing an alternative to chemical synthesis, the microbial synthesis approach can be used to predict metabolites in soil, animals and plants prior to conducting metabolism and environmental fate studies. Thus, agrochemical metabolites identified from a small-scale microbial screen can provide a synthetic chemist with information pertinent to the nature of metabolites that would be

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch002

anticipated in metabolism and environmental fate studies. The decision to prepare sufficient quantities (typically grams) of relevant metabolite reference standards by microbial or chemical syntheses can then be determined based on a variety of considerations such as feasibility, time and cost.

The important benefits of the *in vitro* microbial approach can be illustrated with a few examples taken from the literature. Cyprodinil (4-cyclopropyl-6-methyl-*N*-phenyl-2-pyrimidinamine) is a fungicide that exhibits a broad-spectrum activity against a variety of phytopathogenic fungi on cereals, grapes, apples and vegetables. The objective of a microbial transformation study (*3*) was to generate metabolites of this fungicide as well as to predict its environmental fate. A collection of 12 microbial cultures, all known to contain cytochrome P-450 monooxygenases or other degradative enzymes, was screened in this study. Metabolites produced in the microbial screen were identified using mass spectrometry and ^{1}H-NMR as well as by chromatographic comparisons to authentic reference standards, when available. A representation of metabolite production by the cultures is provided in Figure 1. Nine of the 12 cultures (*Cunninghamella echinulata* var. *elegans* [two different isolates], *C. echinulata* var. *echinulata, Absidia pseudocylindraspora, Streptomyces griseus, S. rimosus, Mucor circinellosides* f. *griseocyanus, Bacillus megaterium*, and *Rhizopus oryzae*) produced a monohydroxylated metabolite (on the phenyl ring in the *para* position to the nitrogen) in yields ranging from 1.2 to 35.6% of the dose (*ca.* 0.1 – 3.6 mg). The filamentous fungus, *B. bassiana*, produced a methylated glycoside of the monohydroxylated metabolite with a yield of 80% (*ca.* 8 mg). Interestingly, a methylated sugar conjugated to a metabolite of the herbicide propham (1-methylethyl phenylcarbamate) was also formed *from B. bassiana* (*4*), suggesting a somewhat unique transformation carried out by this fungal species. In the fungus *A. pseudocylindraspora*, a dihydroxylated metabolite was produced in significant yield whereby hydroxylation occurred on both the phenyl and pyrimidine rings. Dihydroxylated metabolites (on the phenyl ring) and a molecular cleavage product were also detected in the broth of the fungus *C. echinulata* var. *elegans*. Interestingly, and most importantly, the two predominant metabolites, the monohydroxylated entity on the phenyl ring, and the dihydroxylated metabolite (i.e., monohydroxylated on both the pyrimidine and phenyl rings) were also implicated in the metabolism of cyprodinil in the rat. That is, in a recent publication (*5*), the sulfate esters of both the monohydroxylated and dihydroxylated microbial metabolites were found in rat urine, thus indicating the strongly predictive value of the microbial transformation approach.

An interesting aspect of the microbial approach for generating sufficient quantities of agrochemical metabolites is the ability to "overload" the microbial cultures. For instance, in the cyprodinil transformations, 10 mg of cyprodinil dissolved in 500 µL of ethanol was added to each 50-mL culture, resulting in a final concentration of 200 mg/L. This concentration well exceeds the water solubility of cyprodinil (16 – 20 mg/L) irrespective of the small amount of co-solvent. However, in practice, over the typical 6 or 7-day incubation period, microbial transformations steadily proceed, removing parent compound from the aqueous medium and converting it to metabolites. As more parent in solution is removed, undissolved

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch002

compound goes into solution and is available for uptake and subsequently is converted to metabolites by the microbial cells. Therefore, at the conclusion of a microbial transformation, relatively insoluble substrates can essentially be quantitatively converted to metabolites. This allows relatively large quantities of metabolites to be produced using the microbial transformation approach. Depending on the culture size and volume of the medium, as well as the number of replicate cultures, milligram to gram quantities of metabolites can be generated.

Clomazone, 2-[(2-chlorophenyl) methyl)]-4,4-dimethyl-3-isoxazolidinone, is a herbicide used against many species of annual broad-leaved and grassy weeds in soybean, pea, oilseed rape, sugar cane, pumpkin and tobacco crops. Metabolism of clomazone in rats, soybeans and soil has been studied and reported (*6-8*). Using the microbial approach, Liu et al. (*9*) screened 41 fungal and bacterial cultures for their ability to generate clomazone metabolites. These cultures represented common soil organisms such as *Absidia, Aspergillus, Bacillus, Candida, Cunninghamella, Curvularia, Mucor, Mycobacterium, Pseudomonas, Rhizopus* and *Streptomyces*. The microbial screen yielded 17 cultures capable of generating clomazone metabolites. Preparative-scale incubations were conducted with the fungi *Aspergillus niger* and *Cunninghamella echinulata,* to generate metabolites in sufficient quantities for spectral identification (EI/MS and ^{1}H-NMR). A representation of metabolites produced by the cultures is provided in Figure 2. Major microbial transformations included hydroxylation and subsequent dehydrogenation at the 5-methylene carbon of the isoxazolidinone ring, hydroxylation of a methyl group on the isoxazolidinone ring, and aromatic hydroxylation at 3$'$. Minor reactions included dihydroxylation, cleavage of the isoxazolidinone N-O bond, and complete removal of the isoxazolidinone ring to form chlorobenzyl alcohol.

The microbial metabolism of clomazone produced most of the metabolites that were also found as aglycones in the metabolism of clomazone by soybeans. Very importantly, the microbial approach was shown to produce the hydroxymethyl metabolite of clomazone which is a particularly difficult-to-synthesize metabolite reference standard.

Another illustrative example of an *in vitro* approach is the microbial production of metabolites from a tetrazolinone herbicide, F5231 ({1-[4-chloro-2-fluoro-5-(ethylsulfonylamino)phenyl]-1,4-dihydro-4-(3-fluoropropyl)-5H-tetrazol-5-one}, a compound that was considered a developmental candidate by the FMC Corporation in the late 1980's (*10*). The intention was that the microbially generated metabolites would predict metabolites produced in the registration-requiring soil, plant and animal metabolism studies. Microbial metabolites could then be produced in larger quantities based on scale-up fermentation procedures. Alternatively, the metabolites produced and identified microbially could be synthesized in larger quantities by conventional chemical synthesis.

The microbial transformations were carried out by the filamentous fungus *Absidia pseudocylindraspora*. Structures of the metabolites as well as a proposed bioconversion pathway are provided in Figure 3. Metabolites, generated in quantities ranging from 1 to 7 mg, were identified using mass, infrared, and nuclear magnetic resonance spectroscopy. The metabolite profile indicated that neither the aromatic

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch002

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch002

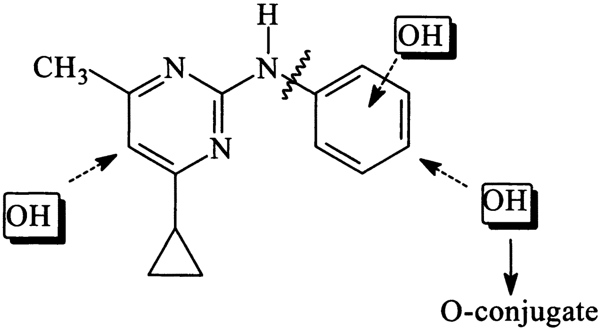


Figure 1. Representation of metabolite production from the microbial transformation of the fungicide cyprodinil. Adapted from [3].

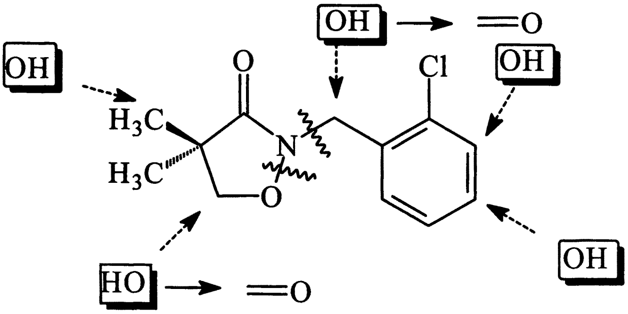
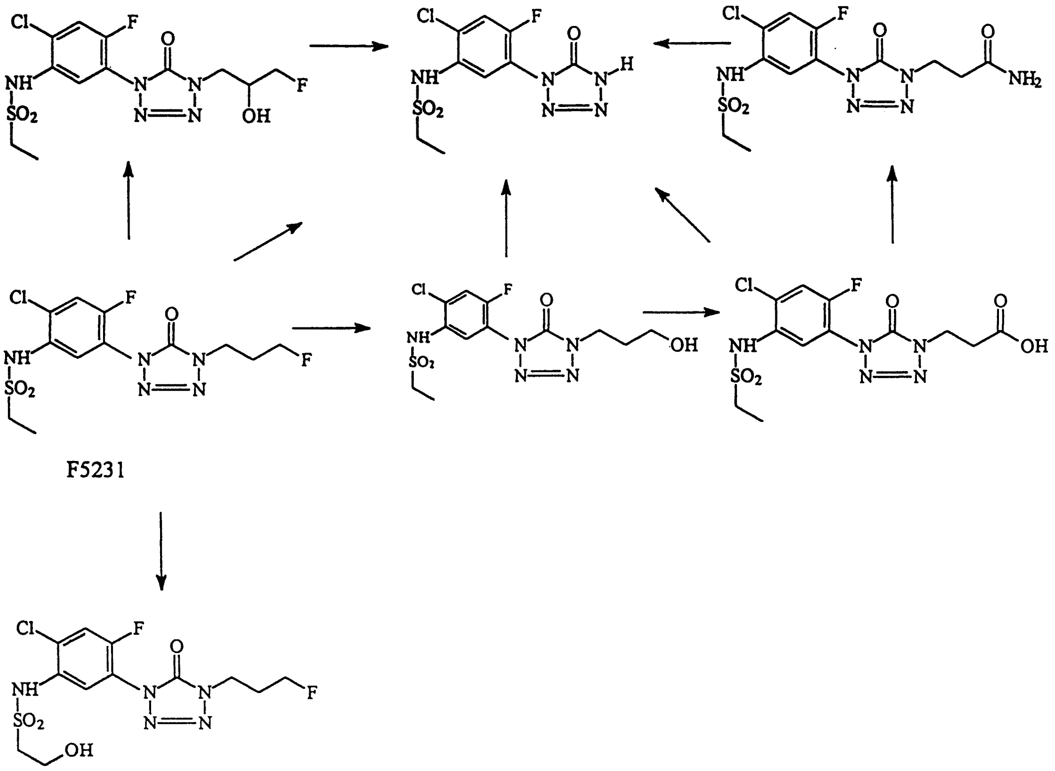


Figure 2. Representation of metabolite production from the microbial transformation of the herbicide clomazone. Adapted from [9].

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch002



F5231

Figure 3. Proposed pathway for bioconversion of the herbicide F5231 by Absidia pseudocylindraspora. Reprinted with permission from [10]. Copyright [1989] American Society for Microbiology.

nor tetrazolinone rings were modified by fungal enzymes. Instead, only side chains (ethylsulfonylamino and fluoropropyl) were attacked. Reactions included various aliphatic hydroxylations, an *N*-dealkylation, oxidation of a primary alcohol to a carboxylic acid and conversion of a carboxylic acid to a carboxamide. In addition, one of the metabolites identified represented cleavage of a carbon-fluorine bond, which was somewhat unexpected given the relative strength of that type of bond. Confirmation of the identity of the defluorinated metabolite was accomplished with ^{19}F-NMR. The authors concluded that the microbially produced metabolites could potentially be used as analytical standards to support animal, plant and soil metabolism studies.

The microbial transformations thus far discussed have utilized whole cells, where suites of enzymes are available for degradation processes. However, isolated microbial enzyme transformations are also very valuable for elucidating degradation pathways and characterizing pathway intermediates that might not be apparent (i.e., not detectable) in whole-cell transformations. Examples pertinent to agrochemicals include hydrolases, dioxygenases, dehalogenases and polyphenoloxidases, to name a few (see Bollag and Dec, this volume).

In vitro methods can also play an important role in facilitating metabolite identification in animals. The value of the approach can be nicely illustrated in a paper that predicted animal metabolism of metolachlor (*11*). In this study, the *in vitro* transformation of metolachlor was studied in rat liver homogenate, namely a cytosolic fraction and a microsomal fraction. In the presence of glutathione, liver cytosolic enzymes catalyzed complete conversion of metolachlor to a more polar metabolite identified as the metolachlor-glutathione conjugate. Liver microsomal enzymes, fortified with NADPH, catalyzed *O*-demethylation, benzylic hydroxylation, and *N*-dealkylation reactions of metolachlor. Eight oxidized metabolites were identified involving single, double and triple hydroxylations at four different sites in the metolachlor molecule (Figure 4). Importantly, the authors concluded that the profile of metabolites produced *in vitro* had been either postulated or observed in metolachlor rat metabolism studies. Also, metabolites produced by rat liver microsomes were nearly identical with those observed by a soil actinomycete (*12*). This observation also provides yet another example of how microbial transformations can serve as models of mammalian metabolism, a concept originally described by Smith and Rosazza (*13*) as M^4 (Microbial Models of Mammalian Metabolism).

I would like to conclude my brief and selective survey of this area by discussing a non-biological approach for generating oxidative metabolites of xenobiotics, similar to those produced by the enzymatic activities of cytochrome P-450 monooxygenase. The work illustrated was conducted by my colleague at Uniroyal Chemical Co., Dr. Hamdy Balba (*14*). The research describes the degradation of a carbamate insecticide, matacil (4-dimethylamino-3-tolyl-*N*-methylcarbamte), by an ascorbic acid oxidation system. The incubation mixture consisted of L-ascorbic acid, ferrous sulfate, EDTA and matacil in phosphate buffer (pH 6.7). The incubation was carried out in an oxygen atmosphere for 2 h in a water bath at 37 °C. The reaction mixture was partitioned with ethyl acetate. The ethyl acetate extracts were combined, concentrated and chromatographically profiled by

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch002

TLC. After separation, metabolites were isolated by preparative TLC and identified by a combination of chromatography and spectroscopy (IR and MS), based on comparisons to reference standards.

The metabolites that were identified (Figure 5) indicated *N*-dealkylation reactions, aromatic ring and methyl group hydroxylation, as well as hydrolysis of the carbamate to the corresponding phenol. Most importantly, these reactions were similar to those observed in plants, insects and rat liver microsomes. Thus, the non-biological "mimic" of the cytochrome P-450 monooxygenases can be quite useful for predicting metabolites produced in biological systems where cytochrome P-450 monooxygenases are widely distributed.

The use of *in vitro* methods in plant metabolism has been extensive, but will not be illustrated in this paper since that area is covered in other chapters of this book by contributions from Dr. Heinrich Sandermann and Dr. Burkhardt Schmidt. In addition, the reader is directed to the excellent *in vitro* plant metabolism papers of Shimabukuro and Walsh (*15*), Mumma and Hamilton (*16*), and Lamoureux and Frear (*17*) that were compiled in a 1978 American Chemical Society-sponsored symposium book. The author is hopeful that the illustrative examples presented in this paper provide a strong sense of the utility of *in vitro* methods, which can support agrochemical metabolism and environmental fate studies.

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch002

References

1. Webster's Seventh New Collegiate Dictionary, G. & C. Merriam Company, Publishers, Springfield, MA, 1976.
2. Schocken, M. J., *J. Ind. Microbiol. & Biotechnol.* **1997**, *19*, 392-400.
3. Schocken, M. J., Mao, J. and Schabacker, D. J., *J. Agric. Food Chem.* **1997**, *45*, 3647-3651.
4. Vigne, B., Archelas, A., Fourneron, J. M. and Furstoss, R., *Tetrahedron* **1986**, *42*, 2451-2456.
5. Müller, T., Thanei, P., Mücke, W., Kriemler, H.-P., and Winkler, T., *Pest. Sci.* **1999**, *55*, 594-596.
6. Wu, J., Creekmore, R. W., Rosen, R. T., and Robinson, R. A., *Abstract of Papers*, 186th National Meeting of the American Chemical Society, Washington, DC, Aug 1983.
7. ElNaggar, S. F., Creekmore, R. W., Schocken, M. J., Rosen, R. T., and Robinson, R. A., *J. Agric. Food Chem.* **1992**, *40*, 880-883.
8. Mervosh, T. L., Sims, G. K., and Stoller, E. W., *J. Agric. Food Chem.* **1995**, *43*, 537-543.
9. Liu, S. Y., Schocken, M. J., and Rosazza, J. P. N., *J. Agric. Food Chem.* **1996**, *44*, 313-319.

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch002

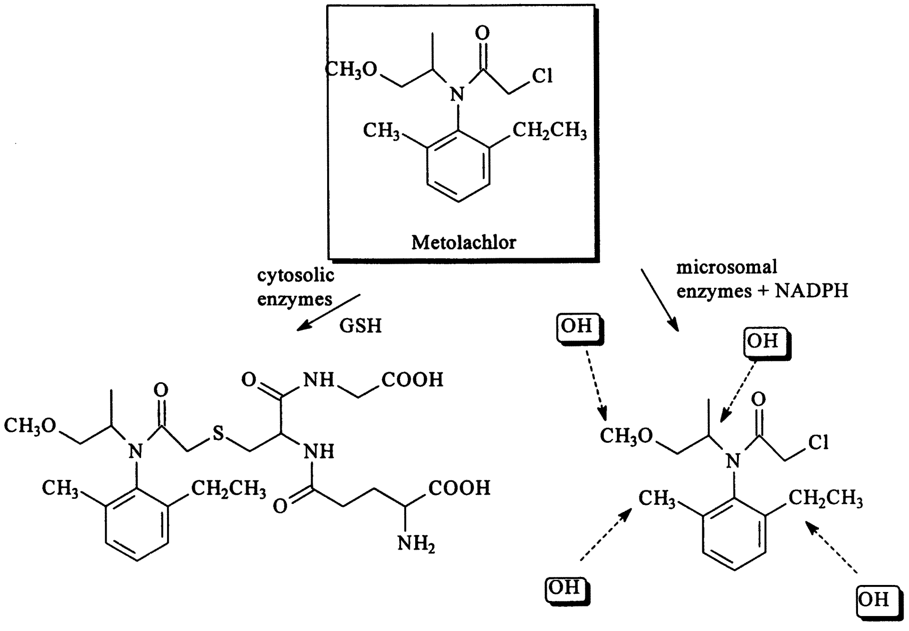

Metolachlor
cytosolic enzymes
GSH
microsomal enzymes + NADPH
OH
OH
OH
OH

Figure 4. Representation of metabolites formed from liver cytosolic and microsomal incubations with the herbicide metolachlor. Adapted from [11].

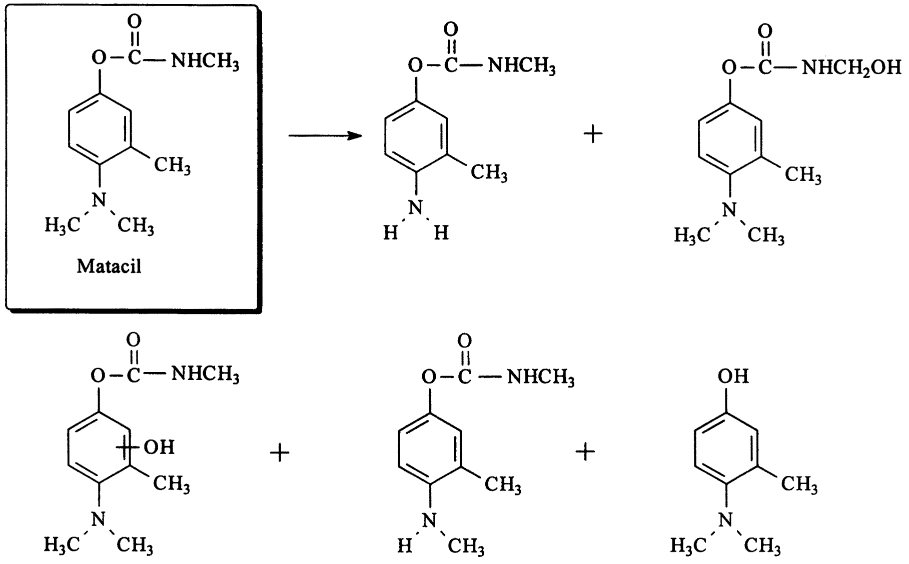


Figure 5. Metabolites produced from the carbamate insecticide matacil by the ascorbic acid oxidation system. Reprinted with permission from [14]. Copyright [1974] Springer-Verlag.

10. Schocken, M. J., Creekmore, R. W., Theodoridis, G., Nystrom, G. J., and Robinson, R. A., *Appl. Environ. Microbiol.* **1989**, *55*: 1220-1222.
11. Feng, P. C. C. and Wratten, S. J., *J. Agric. Food Chem.* **1989**, *37,* 1088-1093.
12. Krause, A., Hancock, W. G., Minard, R. D., Freyer, A. J., Honeycutt, R. C., LeBaron, H. M., Paulson, D. L., Liu, S. Y. and Bollag, J. M., *J. Agric. Food Chem.* **1985**, *33*, 584-589.
13. Smith, R. V. and Rosazza, J. P., *J. Pharm. Sci.* **1975**, *64*, 1737-1753.
14. Balba, M. H. and Saha, J. G., *Bull. Environm. Contam. Toxicol.* **1974**, *11*, 193-200.
15. Shimabukuro, R. H. and Walsh, W. C., In: *Xenobiotic Metabolism: In Vitro Methods;* ACS Symposium Series 97, (Edited by Paulson, G. D., Frear, D. S., and Marks, E. P.), ACS, Washington, D. C., **1979**, 3-34.
16. Mumma, R. O and Hamilton, R. H., ., In*:* *Xenobiotic Metabolism: In Vitro Methods;* ACS Symposium Series 97, (Edited by Paulson, G. D., Frear, D. S., and Marks, E. P.), ACS, Washington, D. C., **1979,** 35-76.
17. Lamoureux, G. L. and Frear, D. S., In*:* *Xenobiotic Metabolism: In Vitro Methods*; ACS Symposium Series 97, (Edited by Paulson, G. D., Frear, D. S., and Marks, E. P.), ACS, Washington, D. C., **1979**, 77-128.

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch002

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch003

Chapter 3

Metabolic Profiling Using Plant Cell Suspension Cultures

Burkhard Schmidt

Department of Biology V (Ecology/Ecotoxicology/Ecochemistry), Technical University Aachen, D-52056 Aachen, Germany

Cell cultures of higher plants are often used to investigate plant metabolism of xenobiotics. Such cell systems are thought to represent the intrinsic enzymatic capacity of the respective species. Mostly, metabolic rates are more rapid in cell cultures, whereas formation of bound residues is usually higher in intact plants. In Germany, a standardized cell culture test procedure was developed and employed to determine metabolic rates of pesticides which were regarded as applicable within the framework of guidelines for pesticide registration. On the basis of recent data, the usefulness of the procedure is discussed. Cultured plant cells are convenient systems for screening and for the prediction of the metabolic patterns of xenobiotics in different species, and of the soluble metabolites to be expected in plants in general. The benefits of this novel conception of metabolic profiling using a set of plant cell suspension cultures is illustrated by means of experiments performed with the insecticide parathion, the herbicide atrazine, the polyaromatic hydrocarbon pyrene, and the non-selective herbicide glufosinate.

During the past thirty years, axenic cell suspension and callus cultures of higher plants have been used to support investigations on the metabolism of pesticides and xenobiotics. The objectives of studies using cell cultures were to identify and quantify the soluble metabolites formed, to quantify and characterize the portions of non-extractable residues formed, to evaluate metabolic rates of xenobiotics, to determine differences among metabolic patterns of pesticides produced by different plant species, to explore the enzymology and genetics of individual biotransformation reactions, and to produce on a large scale the xenobiotic metabolites needed for other studies. Plant cell cultures have also been used to investigate the phytotoxicity of pesticides, and the mechanisms of action of herbicides

In Pesticide Biotransformation in Plants and Microorganisms; Hall, J., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2000.

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch003

and growth regulators. Comprehensive reviews covering all of these aspects have been published previously (*1-13*).

Without doubt, all investigations on the plant metabolism of xenobiotics using cultured plant cells have considerably promoted our basic understanding in this field. In addition, a number of studies were thought to contribute to the evaluation of the environmental fate of the foreign compounds. Plant cell cultures however, are artificial systems when compared to intact plants grown under environmental conditions. Thus, the relevance of the data obtained from these model systems is considered as uncertain in some cases. The present paper will address two topics: First, the evaluation of metabolic rates of pesticides by application of a standardized plant cell culture metabolism test will be discussed. The second topic deals with the determination of metabolic pathways of xenobiotics using a standard set of plant cell suspension cultures. This novel conception of metabolic profiling was developed in the last years. Regarding both topics, special attention will be given to environmental relevance.

Maintainance and Characterization of Plant Cell Cultures

Plant cell cultures are initiated from aseptic explants which can be produced, *e.g.*, from leaves, hypocotyls or roots. The detached plant parts are sterilized consecutively with ethanol and sodium hypochlorite solution. Then, the plant material is cut into pieces and placed on a solidified medium. After several weeks, the callus tissue formed can be removed. Plant cells can be grown either as callus cultures on a solidified medium or as suspensions in liquid media. Suspension cultures consist of cell aggregates, plus single cells. Cells may be cultivated heterotrophically (chlorophyll-deficient in the dark) with an organic carbon source (mainly sucrose), mixotrophically, or fully phototrophically (autotrophically) under light (continuous white light: 19 W m^{-2}, 10,000 lx; CO_2 level: 1 % to 2 %; *14, 15*).

In the author's lab, xenobiotic metabolism studies were performed exclusively using heterotrophic cell suspensions. For most purposes, the cultures were grown in 20 mL of B5 or MS media (*16-19*) contained in 100 mL Erlenmeyer flasks; the plant systems were kept at 27°C in the dark with rotary shaking (110 rpm to 120 rpm). Subcultivation was executed by taking plant material from the preceeding culture using a spoon-shaped sieve (0.8-mm- or 1.0-mm mesh width). Then, 1 g of the tissue was introduced into fresh medium, either by transfer with a spatula, or by passing the cells through the sieve. The latter technique enabled a selection of cell lines with a defined size of cell aggregates. Cell suspensions of both crop and wild plant (weed) species have been employed for xenobiotic metabolism experiments. These were wheat (*Triticum aestivum* L. cv. 'Heines Koga II'; medium: B5, growth regulator: 9.0 µM 2,4-D), soybean (*Glycine max* L. MERR. cv. 'Mandarin'; B5, 4.5 µM 2,4-D), carrot (*Daucus ca-rota* L. cv. 'Maxima'; B5, 0.45 µM 2,4-D), sugarbeet (*Beta vulgaris* ssp. *vulga-ris* var. *altissima* DÖLL; B5, 4.5 µM 2,4-D), foxglove (*Digitalis purpurea* L; 0.45 µM 2,4-D), jimsonweed (*Datura stramonium* L.; MS, 0.45 µM 2,4-D), corn cockle (*Agrostemma githago* L.; MS, 4.5 µM 2,4-D), and common lambsquar-ters (*Chenopodium album* L.; B5, 9.0 µM 2,4-D). The wheat and soybean suspensions were those utilized for the standardized plant cell culture metabolism test.

Plant cell cultures require an exogenous supply of auxin which induces the unorganized proliferation of undifferentiated cells, whereas cytokinins are mostly not essential (*16, 17*). We generally employed the most potent auxin 2,4-D, mainly because the compound can be autoclaved with the media; the respective concentrations used were adjusted empirically in order to obtain high cell densities. Cultured plant cells are commonly regarded as sensitive to shear stress generated by agitation because of their large size, rigid cell walls, and large vacuoles. Shear stress can affect the growth, metabolism, and organization of a cell (*e.g.* wounding and rupture) or a cell aggregate in a negative sense. These effects usually designated as cell damage were mainly discussed with regard to the large scale cultivation of plant cells in fermenters (*20, 21*). Cell damage however, can also occur in shake flasks (*20*); generally, huge differences exist in stress sensitivity among various cell lines (*21*). Thus, shear stress may also affect xenobiotic metabolism studies; these effects are unknown. After termination of experiments with pesticides however, we often observed distinct distributions of transformation products (*e.g.* carbohydrate conjugates) between media and cells. This observation indicates that the cultured plant cells mostly remained intact.

Heterotrophic plant cell suspension cultures can be characterized by a number of external visible attributes, as well as internal qualities and capacities (*22-25*). These traits are mostly derived from the plant itself (species, subspecies, variety), but are noticeably influenced by the explant used for initiation (*e.g.*, root, leaf, hypocotyl), the medium (*e.g.*, growth regulator, carbon source, vitamins), and the method of cultivation (*e.g.*, growth cycle, subcultivation procedure, and temperature). External attributes are, for example, the aggregate size of the suspension, its pigmentation, growth cycle, fresh and dry weights. Internal parameters include pH changes of the medium over the cycle, protein contents of the medium and cells, enzymatic activities of primary and secondary metabolism, degrees of differentiation, lignification and vacuolization, as well as concentrations of secondary metabolites. Specific changes observed with parameters during the growth cycle also contribute to characterization. Regarding all parameters, heterotrophic cell suspensions cultures can be grown reproducibly. However, variations may occur among growth cycles, among parallel suspensions, and over long periods of cultivation (chronological age). These variations are essentially inherent and unavoidable. It should be noted that even minor modifications of the medium or the method of cultivation can affect all these parameters.

Metabolism of Xenobiotics by Heterotrophic Plant Cell Cultures

Heterotrophically cultured plant cells are grown axenically in the dark. Thus, data derived from xenobiotic metabolism experiments using these systems can unequivocally be traced back to the plant tissue, because interfering microbial and photochemical transformations are absent. In addition, the lack of chlorophyll considerably facilitates the extraction, clean-up and identification of pesticide metabolites. As compared to fully phototrophically cultivated cells, hetero-

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch003

trophic plant cell cultures are not injured by moderate concentrations of PS II herbicides. The metabolism of these compounds thus can be studied without undesired phytotoxic effects. However, regarding environmental relevance of the data, fully phototrophic cell cultures are probably more suitable to examine especially the metabolic fate of PS II herbicides. It is generally accepted (with some exceptions) that heterotrophically cultured cells and corresponding intact plants metabolize pesticides and xenobiotics in similar ways, though considerable quantitative differences may be observed. Since cell cultures generally lack penetration and translocation barriers, the systems are thought to represent the intrinsic enzymatic capacity of the plant species. Results derived from metabolism studies with cell suspensions are obtained more rapidly and are more reproducible than in whole plant studies. Often, overall metabolic rates of pesticides are higher in cell cultures but portions of non-extractable (bound) residues formed from xenobiotics are usually higher in intact plants (*3-10*).

Many factors can influence results of pesticide metabolism studies with cell cultures regarding both the quantity and quality of the transformation products formed. Crucial factors include the plant subspecies or variety (*26-28*), the tissue employed for the culture's initiation, the composition of the culture medium (*29-32*), the cultivation conditions, the chronological age (*26, 33-35*), the concentration of the xenobiotic (*36, 37*), and the timing of the pesticide's application during the growth cycle (*38-41*). Most of these factors can be traced back to the specific characteristics and variations of plant cell cultures discussed before.

Often, the metabolism of pesticides in plant tissues is remarkably similar to that observed in (selected) microbial species. Especially, this holds true for the crucial primary transformation products. In addition to plant cell cultures, bacteria and fungi have thus also been used for the elucidation and prediction of metabolic pathways, and in particular for the large scale production of xenobiotic metabolites. This area is covered in another chapter of this book with a contribution by Dr. Mark J. Schocken.

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch003

Evaluation of Metabolic Rates - Standardized Plant Cell Culture Metabolism Test

Encouraged by a number of advantages connected with the utilization of cultured plant cells for xenobiotic metabolism investigations, a cell culture test system using highly standardized suspensions of wheat and soybean was developed by three German research groups in the early 1980s (*8, 10, 42-46*). Wheat and soybean, respectively were thought to represent monocot and dicot species in general. The system was used to study the metabolic fate of ^{14}C-labeled xenobiotics (1 μg mL^{-1}, 8,500 Bq) after incubation periods of 48 h during the late linear growth phase of the cycle. The experiments were terminated by separation of the treated cells from the media. Subsequently, the cells were extracted by a suitable organic solvent (methanol, ethanol, or a methanol/chloroform mixture). Without further clean-up, both the media and cell extracts were analyzed by TLC for the relative amounts of non-metabolized parent pesticide and extractable metabolites formed. The latter were not identified during routine application of the test. Portions of non-extractable residues were determined by combustion of insoluble cell debris. The data resulting from the test system were the metabolic rates of the pesticide in the two cultures, while metabolic rate was defined as the percent-age of the applied ^{14}C-labeled pesticide converted to soluble metabolites and non-extractable residues. One premise of this cell culture metabolism test was that persistence of a pesticide in the suspensions would correlate with persistence of the compound in the plant *in vivo*. Consequently, metabolic rates determined with the test system were regarded as a reasonable contribution to the evaluation of the environmental fate of the compound.

A large number of xenobiotics have been examined with the test procedure (*10, 47*). Table I shows metabolic rates of pesticides obtained from a screening investigation performed during late 1980s (*42, 47*). The wheat and soybean suspensions differed to a certain extent with regard to their enzymatic capacity to transform the pesticides. In 1992, the standardized cell culture metabolism test was integrated by the Federal Biological Research Centre for Agriculture and Forestry (BBA) into the German guidelines for the testing of pesticides (*48*). The applicability of the test in the frame of pesticide registration however, has been discussed controversally. Hitherto, data determined in accordance with the test procedure are not obligatory requirements for the registration of pesticides.

The main findings are the metabolic rates of the pesticide in the wheat and soybean cultures. However, a number of objections can be raised to the standardized plant cell culture metabolism test. It is doubtful whether wheat and soybean are representative of monocot and dicot species, even on the level of plant cell cultures. The wheat and soybean suspensions used may even not be representative of the respective intact plants. Furthermore, it is questionable whether the wheat and soybean varieties and suspensions employed are representative of the respective entire species. Experimental approaches are possible to check if these objections are well-founded. However, difficulties arising are insurmountable due to the large number of relevant plant species and pesticidal compounds. In addition, wheat and soybean represent a monocot and dicot that are very active metabolically, with defined detoxification systems involving cytochromes P450 and glutathione S-transferases. To generalize to other monocots and dicots, in particular to weeds, is considered as an over-generalization, as all other species have much less active and probably different sets of enzymes. Concerning the relevance of metabolic rates derived from plant cell cultures, the reader is referred to recent data on parathion and atrazine presented later.

Ambiguous results are occasionally obtained from the test, which can be avoided by modifications of the procedure: Metabolic rates may show variations among experiments. TLC artifacts simulating high metabolic rates can emerge with media and cell extracts (cf., data of atrazine and pyrene discussed below). Pesticides may not be analyzed reliably by TLC (cf., data of glufosinate discussed below). Metabolism by the plant tissues can result in chemically or enzymatically unstable carbohydrate conjugates of the parent, which are not identified by routine application of the procedure. The environmental relevance of these products considerably differs from that of chemicals detoxified by phase I reactions. So, preliminary data from a metabolism investigation on the herbicide 2,4,5-T showed that in wheat, foxglove and corn cockle, the main metabolic route was conjugation of the parent to its glucosyl ester and processing products. Only minor portions were transformed to 2,4,5-trichlorophenol and a further as yet unidentified phase I metabolite in corn cockle. Regarding their evaluation,

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch003

metabolic rates of chemicals may thus be misleading without metabolite identification.

Table I. Metabolic Rates of Pesticides in Wheat and Soybean Cell Suspension Cultures After 48 h Incubation. Data Were Obtained by Application of the Standardized Plant Cell Culture Metabolism Test

Chemical	*Use*	*Wheat*	*Soybean*
Lindane	I	9.9 (0.6)	11.2 (1.1)
Diflubenzuron	I	10.6 (1.7)	14.6 (6.4)
Monolinuron	H	10.8 (2.4)	22.7 (3.0)
Dimethoate	I, A	11.6 (0.5)	34.6 (2.9)
Metaldehyde	M	12.4 (1.4)	10.7 (0.6)
Atrazine	H	14.0 (1.1)	80.6 (7.6)
Maleic hydrazide	PGR	17.2 (3.4)	80.6 (37.0)
Triadimenol	F	19.3 (2.1)	21.4 (2.2)
Diquat	H	24.8 (21.6)	25.6 (21.3)
Methoxychlor	I	37.4 (2.9)	52.7 (17.6)
Carbaryl	I	38.3 (11.3)	62.5 (22.4)
2,4,5-T	H	46.0 (3.2)	11.3 (1.2)
Chlorpropham	H	48.2 (22.9)	78.0 (26.0)
Endosulfan	I	50.7 (2.3)	85.6 (1.6)
Aldicarb	I, A, N	89.8 (0.5)	93.6 (1.7)
Pentachlorophenol	I, F, H	90.7 (18.7)	60.2 (6.9)
Captan	F	90.8 (0.8)	86.7 (1.1)
Folpet	F	91.6 (7.3)	97.0 (8.4)
2,4-D	H	92.8 (17.7)	21.5 (3.5)
Parathion	I	93.0 (38.7)	77.4 (3.6)
Dichlofluanide	F	93.7 (7.9)	97.9 (7.9)
Malathion	I, A	94.4 (6.8)	88.4 (11.4)
Tetraethylthiuram disulfide	F	98.0 (15.1)	85.6 (8.6)

NOTE: Units of metabolic rates are % of applied ^{14}C (1 µg mL^{-1}) after 48 h incubation; values in parenthesis are portions of non-extractable residues. A = acaricide, F = fungicide H = herbicide, I = insecticide, M = mulluscicide, N = nematicide, PGR = plant growth regulator.

SOURCE: Data are from references *42, 47*.

Determination of Metabolic Patterns - Metabolic Profiling

Two important observations can be derived from pesticide metabolism studies with plant cell suspension cultures. First, if a pesticide is transformed by a cell culture, the plant species has the enzymatic capacity to metabolize the chemical. Secondly, since in most cases the same metabolites are found in cell cultures and plants, cell suspensions are suitable systems to demonstrate the metabolic patterns of a pesticide produced by different species. These two statements are the basis of our novel conception of metabolic profiling, *i.e.*, a set of cell suspension cultures (five to ten) including crop and wild plant species can be utilized to ob-

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch003

tain a qualitative approximation of the pesticide's metabolism expected in the plant kingdom.

Thus, the basic procedure of the standardized plant cell culture metabolism test can be modified by altering incubation and treatment parameters. This includes extending incubation periods, modifying timing of application during the growth cycle, and increasing amounts of ^{14}C applied per assay. Furthermore, the basic procedure has to be supplemented with appropriate clean-up and analytical methods in order to identify soluble metabolites. We have employed various clean-up methods, such as extraction of media and concentrated cell extracts with *n*-butanol, preparative TLC and chromatography on Sephadex LH-20. The purpose of the clean-up was to obtain plant extracts that could be analyzed more easily and reliably. Final identification of the pesticide metabolites was carried out by enzymatic and chemical hydrolysis of carbohydrate conjugates, radio-TLC, radio-HPLC, GC, GC-MS, and LC-MS. Identification of metabolites was facilitated, when major metabolites were previously known from studies with plants, and were available as reference compounds.

Using cell suspension cultures of both crop and wild plants, we performed metabolic profiling investigations with the insecticide parathion (*49*), the herbicide atrazine (*50*), the polyaromatic hydrocarbonc pyrene (*51*), and the herbicide glufosinate. The results of these investigations and the benefits of metabolic profiling using a set of plant cell suspension cultures will be discussed.

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch003

Parathion

Metabolic rates and patterns of the insecticide parathion (uniformly ^{14}C-ring-labeled) were examined in cell suspension cultures of wheat, soybean, carrot, foxglove, jimsonweed, and a second soybean cultivar, var. 'Harosoy 63' grown in Miller (M) medium (*52*), plus growth regulators kinetin and 1-naphthaleneacetic acid. The metabolic rates are shown in Table II. Portions of soluble transformation products were determined by TLC of media and cell extracts, and confirmed after *n*-butanol extraction. Conjugates were identified following cleavage by chemical and enzymatic hydrolysis. All metabolites were isolated by preparative TLC, identified by TLC and GC-MS.

In all cell suspensions investigated, the same metabolic pattern emerged. In addition to the parent parathion, its oxon derivative, 4-nitrophenol, the 4-nitrophenol glucoside and lower amounts of other glycosides were found (Figure 1). The respective carbohydrate conjugation pattern depended to a certain extent on the plant species. On the whole, the data unequivocally proved that higher plants, generally possess the enzymatic capacity to biotransform parathion. This hydrolysis of the organophosphorus bond was not unequivocally proven, although it was reported (*49, 53*). However, these published results did not clearly distinguish between microbial and plant metabolism, or abiotic reactions. From metabolic profiling, we concluded that the environmentally relevant plant metabolites of parathion are paraoxon and 4-nitrophenol.

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch003

Table II. Metabolic Profiling of Parathion, Atrazine and Pyrene Using Plant Cell Suspension Cultures: Metabolic Rates

Plant Species	*Parathion* [a]	*Atrazine* [b]	*Pyrene* [c]
Wheat	94.4 (38.3)	30.3 (4.7)	86.1 (38.3) [d]
Soybean 'Mandarin'; B5	87.9 (9.4)	48.8 (3.6)	6.4 (6.4) [e]
Soybean 'Harosoy 63'; M	24.2 (2.5)	44.3 (3.8)	-[f]
Soybean 'Harosoy 63'; B5, coarse	-[f]	12.1 (2.0)	-[f]
Soybean 'Harosoy 63'; B5, fine	-[f]	80.4 (8.9)	-[f]
Carrot	32.8 (0.9)	90.8 (2.8)	-[f]
Foxglove	38.2 (3.2)	27.7 (2.1)	8.8 (2.3
Jimsonweed	37.3 (7.8)	17.8 (1.9)	7.2 (7.2)
Corn cockle	-[f]	70.4 (4.2)	-[f]

NOTE: Units of metabolic rates are % of applied ^{14}C (1 μg mL^{-1}); values in parenthesis are portions of non-extractable residues. Portions of soluble metabolites were determined by [a] TLC of media and cell extracts, [b] TLC after *n*-butanol extraction of media and cell extracts, and [c] TLC and HPLC after clean-up of media and cell extracts. Incubation was 48 h, except [d] 14 days and [e] 4 days. [f] Experiment was not executed.
SOURCE: Data are from references *49-51*.

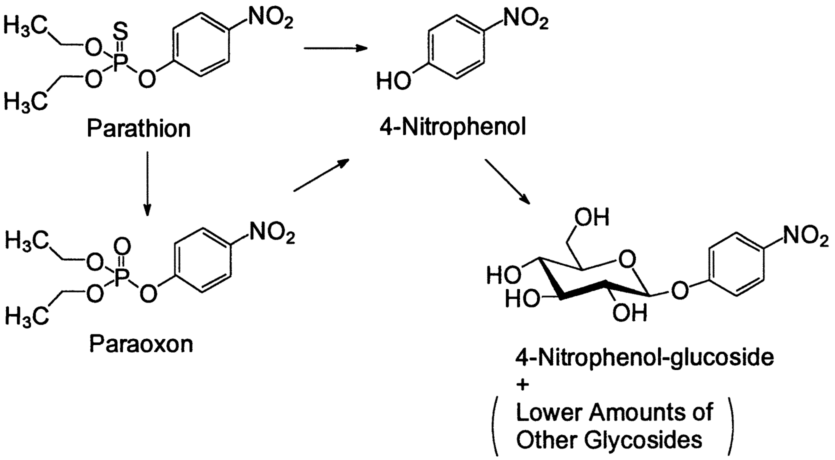


Figure 1. Metabolic profiling of parathion using plant cell suspension cultures. Metabolites were identified by TLC and GC-MS. (Data are from reference 49).

Atrazine

Metabolic profiling experiments with the herbicide atrazine (^{14}C-labeled heterocyclic ring) were executed with cell suspensions of wheat, soybean, carrot, foxglove, jimsonweed, and corn cockle. Addionally, three other soybean cultivars were examined: soybean var. 'Harosoy 63' (medium: M; growth regulators: kinetin and 1-naphthaleneacetic acid), soybean var. 'Harosoy 63' (B5; 2,4-D, coarse cell aggregates), and soybean var. 'Harosoy 63' (B5; 2,4-D, fine cell

American Chemical Society Library

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch003

aggregates due to subcultivation by sieving). Media and cell extracts were analyzed by TLC for portions of soluble transformation products. Both fractions were subsequently extracted with *n*-butanol; organic extracts and remaining aqueous phases were re-examined by TLC. In soybean, soybean var. 'Harosoy 63' (B5, coarse), and jimsonweed, noticeable differences were observed among *n*-butanol extracts and corresponding media and cell extracts regarding portions of non-metabolized atrazine. Differences were attributed to TLC artifacts emerging with media and cell extracts due to adsorption trapping of the parent to natural plant constituents present at the origin of the TLC plates. Thus, distinctly higher metabolic rates were miscalculated. The metabolic rates shown in Table II were derived from TLC data after *n*-butanol extraction. For identification of soluble transformation products, the radioactivity contained in *n*-butanol extracts and aqueous phases was separated by preparative TLC. Resulting fractions were further analyzed by TLC and GC-MS.

Due to the availability of reference compounds, metabolic profiling of atrazine focussed upon deethyl-, deisopropyl- and dethyldeisopropyl-atrazine, as well as hydroxyatrazine. Only the highly polar metabolites formed were quantified. Results (Figure 2) showed that different metabolic patterns of atrazine were obtained. Dethyl-atrazine was detected in all cultures examined, whereas deiso propyl-atrazine could only be identified in wheat, carrot and corn cockle.

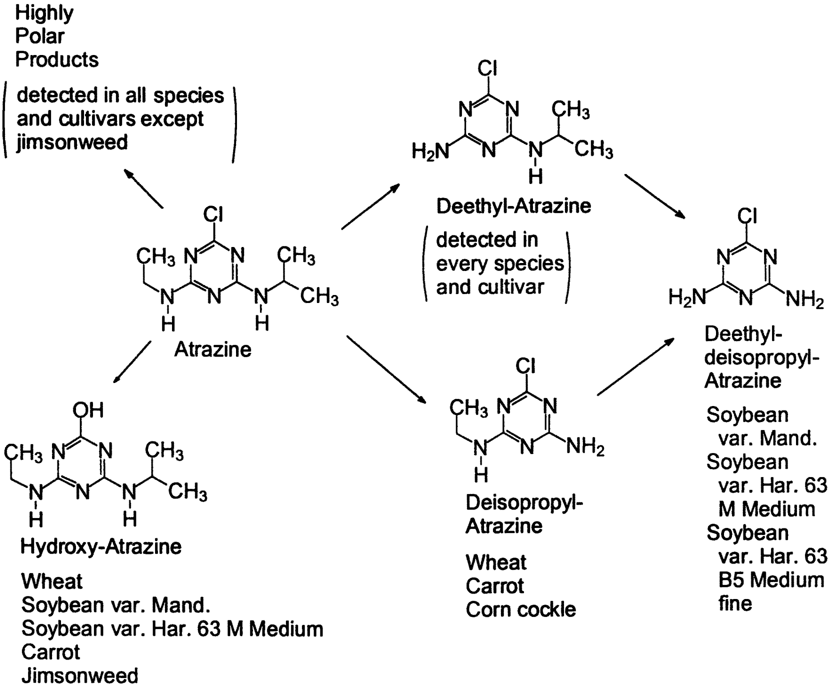

Highly
Polar
Products
detected in all species and cultivars except jimsonweed
Atrazine
Deethyl-Atrazine
detected in every species and cultivar
Deethyl-deisopropyl-Atrazine
Soybean var. Mand.
Soybean var. Har. 63 M Medium
Soybean var. Har. 63 B5 Medium fine
Deisopropyl-Atrazine
Wheat
Carrot
Corn cockle
Hydroxy-Atrazine
Wheat
Soybean var. Mand.
Soybean var. Har. 63 M Medium
Carrot
Jimsonweed

Figure 2. Metabolic profiling of atrazine using plant cell suspension cultures. Percentages of metabolites shown were > 5 % of applied ^{14}C; metabolites were identified by TLC and GC-MS. (Data are from reference 50).

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch003

The completely *N*-dealkylated metabolite was only found in soybean. With the excep-tion of jimsonweed, highly polar metabolites were found with every suspension studied. The findings of metabolic profiling suggest that in plants, relevant pri-mary metabolites of atrazine are dealkylated products and hydroxyatrazine. Concerning the scope of metabolites examined, the investigation was limited; the potential role and activity of glutathione conjugation was not assessed. Nev-ertheless, within this limited scope the findings showed that a reasonable meta-bolic scheme of atrazine can be obtained from a set of cell suspensions (*54*).

Pyrene

The investigation with pyrene (^{14}C-labeled at positions 4, 5, 9 and 10 of the polynuclear system) was performed using wheat, soybean, foxglove and jimsonweed. For determination of portions of soluble metabolites, media and cell extracts were analyzed by TLC. All cell extracts were then subjected to preparative TLC; separated ^{14}C fractions were re-analyzed by TLC and then analyzed by HPLC. With soybean and jimsonweed, this procedure revealed artifacts during TLC examination of the cell extracts, simulating higher metabolic rates. Artifacts were not observed with wheat and foxglove. The metabolic rates of pyrene displayed in Table II were derived after clean-up. In the suspensions of soybean and jimsonweed, no soluble metabolites were detected. Since the bound residues were not examined, it remained unclear whether pyrene was metabolized by these two species. Pyrene's metabolic rates in the suspensions suggest that the enzymatic capacity to transform the xenobiotic is absent or extremely low in a number of species.

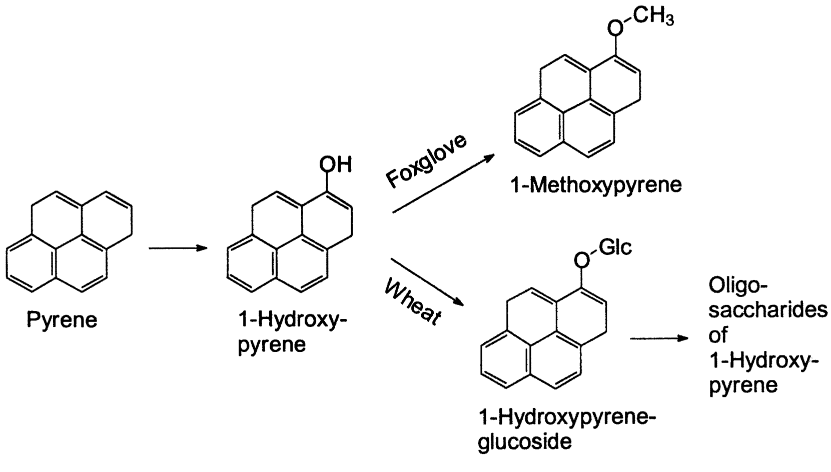

O-CH3
Foxglove
1-Methoxypyrene
OH
Pyrene
1-Hydroxy-pyrene
Wheat
O-Glc
Oligo-saccharides of 1-Hydroxy-pyrene
1-Hydroxypyrene-glucoside

Figure 3. Metabolic profiling of pyrene using plant cell suspension cultures. Metabolites were identified by TLC, GC-MS and HPLC. (Data are from reference 51).

In wheat and foxglove, the only primary metabolite found was 1-hydroxypyrene (Figure 3). This compound was not present in free form. In wheat, 1-hydroxypyrene was transformed to a mixture of glycosyl conjugates; the initial conjugate appeared to be the glucoside. In the foxglove cell suspensions, the only metabolite detected was 1-methoxypyrene. After isolation by preparative TLC, the latter product was identified by TLC, HPLC and GC-MS. In the case of wheat, the carbohydrate conjugates were separated by preparative TLC and the resulting ^{14}C fractions were subjected to hydrolysis. The xenobiotic aglycone was identified by TLC, HPLC and GC-MS. Thus, our results proved that pyrene can be transformed by plant tissues, and suggest that the relevant phase I metabolite is 1-hydroxypyrene. This finding agrees with field data published in Japan (*55*) where authors reported that 1-hydroxypyrene was present in the leaves of various tree species in the form of glucosides and glucuronides. Although 1-hydroxypyrene was supposedly derived from the parent xenobiotic ubiquitously present in the environment, detailed routes remained unclear. Possibly, 1-hydroxypyrene was formed microbially or abiotically outside the plants. The authors also speculated that both hydroxylation and conjugation of pyrene were catalyzed by plant enzymes in the leaves. Our findings indicate that the latter route contributes to the formation of carbohydrate conjugates of 1-hydroxypyrene. Due to its high K_{OW}, pyrene is intensely sorbed to soil organic matter, resulting in decreased bioavailability. The compound is not taken up by roots and translocated in plants (*51*, *56*, *57*). In contrast, uptake of airborne pyrene from vapor phase or particulate fraction was regarded as an important process. The polyaromatic hydrocarbon partitions into the inert waxy cuticle and can be absorbed by the symplast of the leaves (*51*, *56*, *58*, *59*), where metabolic enzymes (*e.g.*, cytochromes P450 and glycosyltransferases) are present. Thus, we concluded that our *in vitro* systems pointed to the relevant plant metabolite of pyrene.

Glufosinate

Metabolic profiling studies with the herbicide glufosinate were executed using cell suspension cultures of carrot, foxglove, jimsonweed, genetically unmodified sugarbeet, and the corresponding transgenic (*bar*-gene), glufosinate-resistant sugarbeet. Experiments with carrot and sugarbeet were performed with the L-enantiomer of glufosinate (LGA), whereas foxglove and jimsonweed were treated with a racemic mixture of the herbicide (GA). Chemicals contained a ^{14}C-label at positions 3 and 4. Glufosinate was absorbed by the cells only to a low extent (carrot: 21.1 %, foxglove: 17.2 %, jimsonweed: 8.5 %, sugarbeet: 4.3 %, transgenic sugarbeet: 3.6 % of applied ^{14}C). TLC and HPLC analysis of media and (aqueous) cell extracts was unsuccessful. Consequently, media and cell extracts were subjected to clean-up. Then, portions and identity of the soluble metabolites of glufosinate were determined by HPLC. Transformation products of the herbicide were found in cell extracts of carrot, foxglove, jimsonweed and trangenic sugarbeet; portions were low. Since amounts of non-extractable residues were also negligible, extremely low metabolic rates of glufosinate were obtained (Table III). Expected differences between the transgenic sugarbeet cultivar and remaining species appeared to be absent. It was assumed that cellular uptake influenced glufosinate's metabolism. All data were thus re-calculated.

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch003

Based on absorbed ^{14}C, the metabolic rate of glufosinate in transgenic sugarbeet was above 60 %, and differed from those in the unmodified species (Table III). In the untransformed species, low metabolic rates of the herbicide were expected. For some time the compound was known not to be metabolized by plants. Low metabolic rates of glufosinate were also reported for suspensions of further plant species (*60*). The present results however, unequivocally proved that glufosinate can be metabolized by plant tissues, and corroborated previous data (*60*). Similarly, the plant metabolism of the herbicide glyphosate was demonstrated for the first time using plant cell suspensions (*61*).

Table III: Metabolic Profiling of Glufosinate Using Plant Cell Suspension Cultures: Metabolic Rates After 48 h Incubation

Plant Species	*% of Applied ^{14}C*	*% of Absorbed ^{14}C*
Glufosinate-resistant, transgenic sugarbeet	2.6 (0.4)	65.0 (10.0)
Genetically unmodified sugarbeet	0.3 (0.3)	6.5 (6.5)
Carrot	3.5 (0.1)	16.5 (0.5)
Foxglove	4.7 (0.1)	28.0 (1.7)
Jimsonweed	1.6 (0.3)	16.3 (1.2)

NOTE: Cell suspensions were treated with 1 µg mL^{-1} glufosinate. Values in parenthesis refer to portions of non-extractable (bound) residues. Percentages of soluble metabolites were determined by HPLC after extensive clean-up of media and cell extracts.

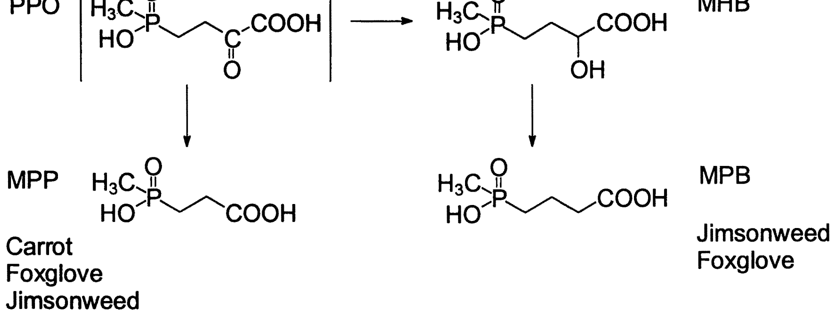


Figure 4. Plant metabolic pathways of glufosinate (data are from references 60, 62-64) and metabolic profiling of glufosinate using plant cell suspension cultures. Metabolites were identified by HPLC after clean-up of cell extracts.

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch003

The metabolic pathways published on glufosinate for genetically unmodified and transgenic, glufosinate-resistant plants are displayed in Figure 4 (*60, 62-64*). In unmodified species, LGA is transformed to the oxo derivative, 2-oxo-4-(hydroxy-methylphosphinyl)-butanoic acid (PPO). This intermediate rapidly decarboxylates spontaneously to the main metabolite, 3-(hydroxymethylphosphinyl)-propionic acid (MPP). PPO can also be reduced to 2-hydroxy-4-(hydroxymethylphosphinyl)-butanoic acid (MHB). The metabolite 4-(hydroxymethylphosphinyl)- butanoic acid (MPB) was only identified in plant cell cultures and is regarded as metabolic artifact of these systems. In transgenic plants, LGA is acetylated to non-phytotoxic *N*-acetyl L-glufosinate (NAG); trace amounts of 'non-transgenic' metabolites are occasionally formed. The metabolic patterns observed in the present profiling study are included in Figure 4. In carrot, foxglove and jimson-weed, MPP was identified as main metabolite, whereas NAG was the only meta-bolite found in transgenic sugarbeet. Additionally, MPB emerged in trace amounts in foxglove and jimsonweed. No soluble metabolites were found in un-modified sugarbeet, and no 'non-transgenic' metabolites emerged in the trans-genic cultivar. Furthermore, MHB and PPO were generally not detected. In con-trast to the relative ease of cell suspension studies conducted on parathion, atra-zine and pyrene, the highly polar herbicide glufosinate was very problematic. This was due to low cellular absorption, low metabolic rate, and unusual physi-cochemical properties of the compound. Glufosinate thus clearly demonstrated the experimental limits of plant cell suspension cultures for metabolism studies. Despite these difficulties, reasonable findings resulted from metabolic profiling. In excised leaves of carrot and foxglove, we showed that glufosinate was exclu-sively transformed to MPP. With the exception of MPB (foxglove suspensions), this result agrees with that derived from the cell cultures.

Table IV: Metabolic Profiling of Pesticides and Xenobiotics Using Plant Cell Suspension Cultures: Other Published Investigations

Chemical	Plant Species	References
Atrazine	potato, wheat	(*65*)
Bentazone	alfalfa, carrot, maize, potato, rice, soybean, etc.	(*66*)
p-Chlorophenyl-methylsulfide	carrot, cotton, tobacco	(*67*)
Cyfluthrin	apple, carrot, cotton, peanut, potato, tomato, etc.	(*68*)
2,4-D	carrot, jackbean, maize, sunflower, tobacco	(*69*)
DDT	soybean, wheat	(*70*)
EPTC	maize, peanut	(*71*)
Formaldehyde	*Chlorophytum comosum*, soybean	(*72*)
Glufosinate	maize, soybean, wheat	(*60*)
Glyphosate	maize, soybean, wheat	(*61*)
4-*n*-Nonylphenol	barley, carrot, soybean, tomato, wheat, etc.	(*37*)
Terbutryne	potato, wheat	(*65*)

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch003

Conclusions

A useful approach to evaluate the metabolism of pesticides in agriculturally relevant plant species is *via* plant cell suspension cultures. Metabolic profiling studies using a set of cell suspensions should be performed complementary to experiments with whole or intact plants. According to the present results and previously published data (Table IV), benefits from such studies are: a rapid survey of the plant metabolism of the pesticide at early stages of development of a pesticide candidate, a greater data base of the plant metabolism, a reasonable confirmation of results obtained with relevant plants, and information on crucial metabolites not formed in relevant plants. Thus, metabolic profiling using plant cell suspensions is considered to be a useful contribution for the evaluation of pesticide environmental fate.

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch003

References

1. Barz, W. In *Plant Tissue Culture and Its Bio-technological Application*; Barz, W.; Reinhard, E.; Zenk, M. H., Eds.; Springer-Verlag: Berlin, 1977; pp 153-171.
2. Sandermann, H.; Diesperger, H.; Scheel, D. In *Plant Tissue Culture and Its Bio-technological Application*; Barz, W.; Reinhard, E.; Zenk, M. H., Eds.; Springer-Verlag: Berlin, 1977; pp 178-196.
3. Shimabukuro, R. H.; Walsh, W. C. In *Xenobiotic Metabolism: In Vitro Methods*; Paulson, G. D.; Frear, D. S.; Marks, E. P., Eds.; ACS Symposium Series 97; American Chemical Society: Washington DC, 1979; pp 3-34.
4. Mumma, R. O.; Hamilton, R. H. In *Xenobiotic Metabolism: In Vitro Methods*; Paulson, G. D.; Frear, D. S.; Marks, E. P., Eds.; ACS Symposium Series 97; American Chemical Society: Washington DC, 1979; pp 35-76.
5. Mumma, R. O.; Hamilton R. H. *J. Toxicol. Clin. Toxicol.* **1982-83**, *19*, 535-555.
6. Mumma, R. O.; Davidonis, G. H. In *Progress in Pesticide Biochemistry and Toxicology*; Hutson, D. H.; Roberts, T. R., Eds.; Wiley: Chichester, 1983; Vol. 3, pp 255-278.
7. Sandermann, H; Scheel, D.; v.d. Trenck, T. *J. Appl. Polymer Sci. Appl. Polymer Symp.* **1983**, *37*, 407-420.
8. Swisher, B. A. In *Biotechnology and Agricultural Chemistry*; LeBaron, H. M.; Mumma, R. O.; Honeycutt, R. C.; Duesing, J. J., Eds.; ACS Symposium Series 334; American Chemical Society: Washington DC, 1987; pp 18-40.
9. Camper, N. D.; McDonald, S. K. *Rev. Weed Sci.* **1989**, *4*, 169-190.
10. Komoßa, D.; Langebartels, C.; Sandermann, H. In *Plant Contamination: Modelling and Simulation of Organic Chemical Processes*. Trapp, S.; McFarlane, C., Eds.; CRC Press: Boca Raton, 1995; pp 69-103.
11. Gressel J. In *Advances in Cell Culture*; Maramorosch, K., Ed.; Academic Press: Orlando, 1984; Vol. 3, pp 93-181.
12. Gressel J. In *Biotechnology and Agricultural Chemistry*; LeBaron, H. M.; Mumma, R. O.; Honeycutt, R. C.; Duesing, J. J., Eds.; ACS Symposium Series 334; American Chemical Society: Washington DC, 1987; pp. 41-52.

13. Grossmann, K. In *Advances in Cell Culture*; Maramorosch, K.; Sato, G. H., Eds.; Academic Press: Orlando, 1988; Vol. 6, pp 90-136.
14. Hüsemann, W. In *Cell Culture and Somatic Cell Genetics of Plants*; Vasil, I. K., Ed.; Academic Press: Orlando, 1984; Vol. 1, pp 182-191.
15. Hüsemann, W. In *Cell Culture and Somatic Cell Genetics of Plants*; Vasil, I. K., Ed.; Academic Press: Orlando, 1985; Vol. 2, pp 213-252.
16. Dixon, R. A. In *Plant Cell Culture - a Practical Approach*; Dixon, R. A., Ed.; IRL Press: Oxford, 1985; pp 1-20.
17. Ozias-Akins, P.; Vasil, I. K. In *Cell Culture and Somatic Cell Genetics of Plants*; Vasil, I. K., Ed.; Academic Press: Orlando, 1985; Vol. 2, pp 129-147.
18. Gamborg, O. L., Miller, R. A., Ojima, K. *Exp. Cell Res.* **1968**, *50*, 151-158.
19. Murashige, T., Skoog, F. *Physiol. Plant.* **1962**, *15*, 473-497.
20. Scragg, A. H.; Fowler, M. W. In *Cell Culture and Somatic Cell Genetics of Plants*; Vasil, I. K., Ed.; Academic Press: Orlando, 1985; Vol. 2, pp 103-128.
21. Meijer, J. J.; Ten Hoopen, H. J. G.; Luyben, K.C.A.M.; Libbenga, K.R. *Enzyme Microb. Technol.* **1993**, *15*, 234-238.
22. Bollwell, G. P. In *Plant Cell Culture - a Practical Approach*; Dixon, R. A., Ed.; IRL Press: Oxford, 1985; pp 107-126.
23. Fukuda, H.; Komamine, A. In *Cell Culture and Somatic Cell Genetics of Plants*; Vasil, I. K., Ed.; Academic Press: Orlando, 1985; Vol. 2, pp 150-212.
24. Lindsey, K.; Yeoman, M. M. In *Cell Culture and Somatic Cell Genetics of Plants*; Vasil, I. K., Ed.; Academic Press: Orlando, 1985; Vol. 2, pp 61-101.
25. Schripsema, J.; Verpoorte, R. *J. Nat. Prod.* **1995**, *58*, 1305-1314.
26. Davidonis, G. H.; Hamilton, R. H.; Mumma, R. O. *Plant Physiol.* **1980**, *65*, 94-97.
27. Abusteit, E. O.; Corbin, F. T.; Schmitt, D. P.; Burton, J. W.; Worsham, A. D.; Thompson, L. *Weed Sci.* **1985**, *33*, 618-628.
28. Menéndez, J.; De Prado, R. *Physiol. Plant.* **1997**, *99*, 97-104.
29. Brain, K. R.; Lines, D. S. In *Plant Tissue Culture and Its Bio-technological Application*; Barz, W.; Reinhard, E.; Zenk, M. H., Eds.; Springer-Verlag: Berlin, 1977; pp 197-203.
30. Montague, M. J.; Enns, R. K.; Siegel, N. R.; Jaworski, E. G. *Plant Physiol.* **1981**, *67*, 701-704.
31. Cole, D. J.; Owen, W. J. *Pestic. Sci.* **1987**, *19*, 67-78.
32. Lewer, P.; Owen, W. J. *Pestic. Biochem. Physiol.* **1989**, *33*, 249-256.
33. Cole, D. J.; Owen, W. J. *Pestic. Biochem. Physiol.* **1987**, *28*, 354-361.
34. Davidonis, G. H.; Hamilton, R. H.; Mumma, R. O. *Plant Physiol.* **1978**, *62*, 80-83.
35. Edwards, R.; Owen, W. J. *Planta* **1988**, *175*, 99-106.
36. Schneider, B.; Schütte, H. R.; Günther, G.; Köhler, S. *J. Plant Physiol.* **1987**, *127*, 147-152.
37. Bokern, M.; Harms, H. *Environ. Sci. Technol.* **1997**, *31*, 1849-1854.
38. Davis, D. G.; Hodgson, R. H.; Dusbabek, K. E.; Hoffer, B. L. *Physiol. Plant.* **1978**, *44*, 87-91.
39. Langebartels, C.; Harms, H. *J. Plant Physiol.* **1984**, *113*, 201-211.
40. Sterling, T. M.; Balke, N. E. *Weed Sci.* **1988**, *36*, 558-563.
41. Canivenc, M. C.; Cagnac, B.; Cabanne, F.; Scalla, R. *Plant Physiol. Biochem.* **1989**, *27*, 193-201.
42. Schuphan, I.; Haque, A.; Ebing, W. *Chemosphere* **1984**, *13*, 301-313.

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch003

43. Haque, A.; Ebing, W.; Schuphan, I. *Chemosphere* **1984**, *13*, 315-320.
44. Ebing, W.; Haque, A.; Schuphan, I.; Harms, H.; Langebartels, C.; Scheel, D.; Von der Trenck, T.; Sandermann, H. *Chemosphere* **1984**, *13*, 947-957.
45. Sandermann, H.; Scheel, D.; Von der Trenck, T. *Ecotox. Environ. Saf.* **1984**, *8*, 167-182.
46. Harms, H.; Langebartels, C. *Plant Sci.* **1986**, *45*, 157-165.
47. Schmidt, B.; Ebing, W.; Schuphan, I. *Gesunde Pflanzen* **1988**, *40*, 245-249.
48. Ebing, W.; Haque, A.; Frost, M.; Schuphan, I.; Schmidt, B.; Harms, H.; Langebartels, C.; Scheel, D.; Von der Trenck, K.; Sandermann, H. *Richtlinien für die Prüfung von Pflanzenschutzmitteln im Zulassungsverfahren: Prüfung des Rückstandsverhaltens - Schnelltest zur Metabolisierbarkeit und zum Abbau von organischen Pflanzenschutzmittelwirkstoffen in pflanzlichen Zellkulturen*; Teil IV, 3-2/1; Biologische Bundesanstalt für Land- und Forstwirtschaft Bundesrepublik Deutschland: Braunschweig, 1992.
49. Van der Krol, D.; Schuphan, I.; Thiede, B.; Schmidt, B. *Pestic. Sci.* **1995**, *45*, 143-152.
50. Schmidt, B.; Siever, M.; Thiede, B.; Breuer, J.; Malcherek, K.; Schuphan, I. *Weed Res.* **1997**, *37*, 401-410.
51. Hückelhoven, R.; Schuphan, I.; Thiede, B.; Schmidt, B. *J. Agric. Food Chem.* **1997**, *45*, 263-269.
52. Diaz-Colon, J. D.; Bovey, R. W.; Davis, F. S.; Baur, J.R. *Physiol. Plant.* **1972**, *27*, 60-64.
53. *Metabolic Pathways of Agrochemicals - Part 2: Insecticides and Fungicides*; Roberts, T.; Hutson, D., Eds.; The Royal Society of Chemistry: Cambridge, 1999; pp 408-416.
54. *Metabolic Pathways of Agrochemicals - Part 1: Herbicides and Plant Growth Regulators*; Roberts, T., Ed.; The Royal Society of Chemistry: Cambridge, 1998; pp. 629-635.
55. Nakajima, D.; Kojima E.; Iwaya S.; Suzuki, I.; Suzuki S. *Environ. Sci. Technol.* **1996**, *30*, 1675-1679.
56. Edwards, N. T. *J. Environ. Qual.* **1983**, *12*, 427-441.
57. Sims, R. C.; Overcash, M. R. *Residue Rev.* **1983**, *88*, 1-68.
58. Simonich, S. L.; Hites, R. A. *Environ. Sci. Technol.* **1994**, *28*, 939-943.
59. Ryan, J. A.; Bell, R. M.; Davidson, J. M.; O'Connor, G. A. *Chemosphere* **1989**, *17*, 2299-2323.
60. Komoßa, D.; Sandermann, H. *Pestic. Biochem. Physiol.* **1992**, *43*, 95-102.
61. Komoßa, D.; Gennity, I.; Sandermann, H. *Pestic. Biochem. Physiol.* **1992**, *43*, 85-94.
62. Dröge-Laser, W.; Siemeling, U.; Pühler, A.; Broer, I. *Plant Physiol.* **1994**, *105*, 159-166.
63. Dröge, W.; Broer, I.; Pühler, A. *Planta* **1992**, *187*, 142-151.
64. Mersey, B. G.; Hall, J. C.; Anderson, D. M.; Swanton, C. J. *Pestic. Biochem. Physiol.* **1990**, *37*, 90-98.
65. Edwards, R.; Owen, W. J. *Pestic. Biochem. Physiol.* **1989**, *34*, 246-254.
66. Sterling, T. M.; Balke, N. E. *Pestic. Biochem. Physiol.* **1989**, *34*, 39-48.
67. Blair, L. C.; Slife, F. W.; Felsot, A.; Plewa, M. J. *Pestic. Biochem. Physiol.* **1984**, *21*, 291-300.
68. Preiß, W.; Wagner, K.; Oehlmann, L.; Engelhardt, G.; Wallnöfer, P. *Chemosphere* **1984**, *13*, 861-872.

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch003

69. Feung, C. S.; Hamilton, R. H.; Mumma, R. O. *J. Agric. Food Chem.* **1975**, *23*, 373-376.
70. Arjmand, M.; Sandermann, H. *Pestic. Biochem. Physiol.* **1985**, *23*, 389-397.
71. Lamoureux, G. L.; Rusness, D. G. *J. Agric. Food Chem.* **1987**, *35*, 1-7.
72. Giese, M.; Bauer-Doranth, U.; Langebartels, C.; Sandermann, H. *Plant Physiol.* **1994**, *104*, 1301-1309.

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch003

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch004

Chapter 4

The Role of Plant and Microbial Hydrolytic Enzymes in Pesticide Metabolism

Robert E. Hoagland and Robert M. Zablotowicz

Southern Weed Science Research Unit, Agricultural Research Service, U.S. Department of Agriculture, Stoneville, MS 38776

Many pesticide molecules containing amide or carbamate bonds, or esters with carbonyl, phosphoryl, and thionyl linkages, are subject to enzymatic hydrolysis. Pesticide hydrolysis by esterases and amidases from plants and microorganisms can serve as a detoxification or activation mechanism that can govern pesticide selectivity or resistance, and initiate or determine the rate of pesticide biodegradation in the environment. Substrate specificity of esterases and amidases varies dramatically among species and biotypes of plants and microorganisms. The constitutive or inducible nature of these enzymes, as well as production of isozymes, is also important in the expression of these mechanisms. For example, increased aryl acylamidase activity has been reported as the mechanism of evolved resistance to the herbicide propanil in two *Echinochloa* weed species [junglerice, *E. colona* (L.) Link; barnyardgrass, *E. crus-galli* (L.) Beauv.]. Other acylamidases, such as a linuron-inducible enzyme produced by *Bacillus sphaericus*, have broad substrate specificities including action on acylanilide, phenylcarbamate, and substituted phenylurea herbicides. Many microbial hydrolytic enzymes are extracellular, thus hydrolysis can occur without uptake. Advances in molecular biology have led to an increased understanding of hydrolytic enzyme active sites, especially those conferring selective specificity. This knowledge will create opportunities for engineering novel resistance mechanisms in plants and biosynthetic and/or degradative enzymes in microorganisms.

In Pesticide Biotransformation in Plants and Microorganisms; Hall, J., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2000.

Hydrolytic enzymes catalyze the cleavage of certain chemical bonds of a substrate by the addition of the components of water (H or OH) to each of the products. Many herbicides, fungicides and insecticides contain moieties (e.g., amide or carbamate bonds, or esters with carbonyl, phosphoryl and thionyl linkages) that are subject to enzymatic hydrolysis. A wide array of hydrolases (amidases, esterases, lipases, nitrilases, peptidases, phosphatases, etc.), with broad and narrow substrate specificities are present in animals, microorganisms and plants. Plants and microorganisms should not necessarily be expected to possess the enzymatic capacity to metabolize xenobiotic compounds. But indeed, it is the multiplicity of certain enzymes and their broad substrate specificities that make pesticide degradation possible. Thus the potential for hydrolytic cleavage of a given pesticide exists in numerous organisms.

Various levels of cellular compartmentalization for these hydrolytic enzymes exist. Some are cytosolic, while others are associated with membranes, microsomes, and other organelles. Some fungi and bacteria also excrete hydrolytic enzymes that act extracellularly on substrates, and thus pesticide detoxification and degradation may occur without microbial uptake of the compound. Certain hydrolases are constitutive, while others are inducible. The ability of an organism to hydrolyze certain pesticides can render the organism resistant to that compound. Many plants are insensitive to various classes of pesticides due to their unique hydrolytic capabilities. Furthermore, differential metabolism is an important mechanism in determining the selective toxicity of a given compound among plants and other organisms. Since molecular oxygen is not involved in hydrolytic activity, hydrolysis can occur under anaerobic and/or aerobic conditions.

In this chapter we examine the hydrolytic transformations of a variety of pesticide chemical classes by plants and microorganisms. The role of these enzymes in pesticide activation, detoxification and degradation is discussed, and opportunities for exploiting novel hydrolytic transformations of xenobiotics are examined.

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch004

Ester Hydrolysis in Plants

Esters are susceptible to hydrolysis by esterases, and to some extent by lipases and proteases. Esterases are ubiquitous in living organisms, and occur as multiple isozymes with varying substrate specificities and catalytic rates. For example, fourteen esterases have been isolated from bean (*Phaseolus vulgaris* L.) and seven from pea (*Pisum sativum* L.) (*1*). The biochemistry and role of plant esterases in xenobiotic metabolism has recently been reviewed (*2*). The physiological role of these esterases may be multifaceted, e.g., they are involved in fruit ripening, abscission, cell expansion, reproduction processes, as well as hydrolysis of ester-containing xenobiotic molecules. Varying degrees of specificity and kinetic rates are observed among plant esterases. For example, an acetyl esterase from mung bean (*Vigna radiata*) hypocotyls hydrolyzed high molecular weight pectin esters (primary physiological substrates), but could more rapidly hydrolyze low molecular substrates such as triactin, and *p*-nitrophenyl acetate (*3*).

Many pesticides are applied as carboxylic acid esters (Figure 1). Since the acid form is generally the active agent, esterases can play a role in pesticide activation, detoxification, and selectivity in plants (Table I.). Many herbicides have been specifically developed as esters to improve absorption into plant tissue, and to alter phytotoxic selectivity. Aliphatic derivatives of the broadleaf herbicide 2,4-D [(2,4-dichlorophenoxy)acetic acid] are widely used. The polar forms of 2,4-D are readily taken up by roots, while long-chain non-polar ester forms (butoxyethyl, and isooctyl esters) are more readily absorbed by foliage. The ability to hydrolyze these 2,4-D esters is widely distributed in sensitive plants such as cucumber (*Cucumis sativus* L.) (*10*) and tolerant plants such as barley (*Hordeum vulgare* L.) (*11*). In a fungicide development program, ester derivatives of the herbicide 4,6-dinitro-*o*-cresol (DNOC) were assessed as fungicides, and for potential phytotoxicity on broadbean (*Vicia faba*) (*12*). Short-chain aliphatic esters (acetate, propionate and isobutyrates) were readily hydrolyzed and were highly phytotoxic. However, aromatic esters of DNOC (chloro- and nitrobenzoates) were hydrolyzed slowly and exhibited low phytotoxicity. The uptake of clopyralid (3,6-dichloro-2-pyridinecarboxylic acid) free acid was greater compared to that of the 1-decyl and 2-ethylhexyl esters in Canada thistle (*Cirsium arvense*) and wild buckwheat (*Polygonum convolvulus*), and in isolated cuticles of *Euonymus fortunei* (*13*, *14*). De-esterification was essential for the 1-decyl and 2-ethylhexyl esters of this herbicide to enter the phloem and translocate to site of action.

Table I. Role of Plant Esterases in Herbicidal Activity in Plants

Mechanism	*Herbicide*	*Plant species*	*Citation*
Activation	Fenoxaprop-ethyl	Wheat, Barley, Crabgrass	(*4*)
	Diclofop-methyl	Wheat, Oat, Wild Oat	(5)
Detoxification	Thifensulfuron-methyl	Soybeans	(*6*)
	Chlorimuron-ethyl	Soybeans	(*7*)
Increased absorption and translocation	Quinclorac esters	Spurge	(*8*)
Increased absorption	2,4-D-Butoxyethyl ester	Bean	(*9*)

The polycyclic alkanoic acid herbicides (PCAs), usually contain more than one ring structure (one is usually a phenyl ring) attached to an asymmetric, non-carbonyl carbon of an alkanoic acid (*15*). Common herbicides in this class include: diclofop-methyl {methyl ester of (±)-2-[4-(2,4-dichlorophenoxy)phenoxy]propanoic acid}, fenoxaprop-ethyl {ethyl ester of (±)-2-[4-[(6-chloro-2-benzoxazolyl)oxy]phenoxy]-propanoic acid}, and fluazifop-butyl {(R)-2-[4-[[5-(trifluoromethyl)-2-pyridinyl]-oxy]phenoxy]propanoic acid}. In plants, PCA-ester hydrolysis, yielding the parent

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch004

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch004

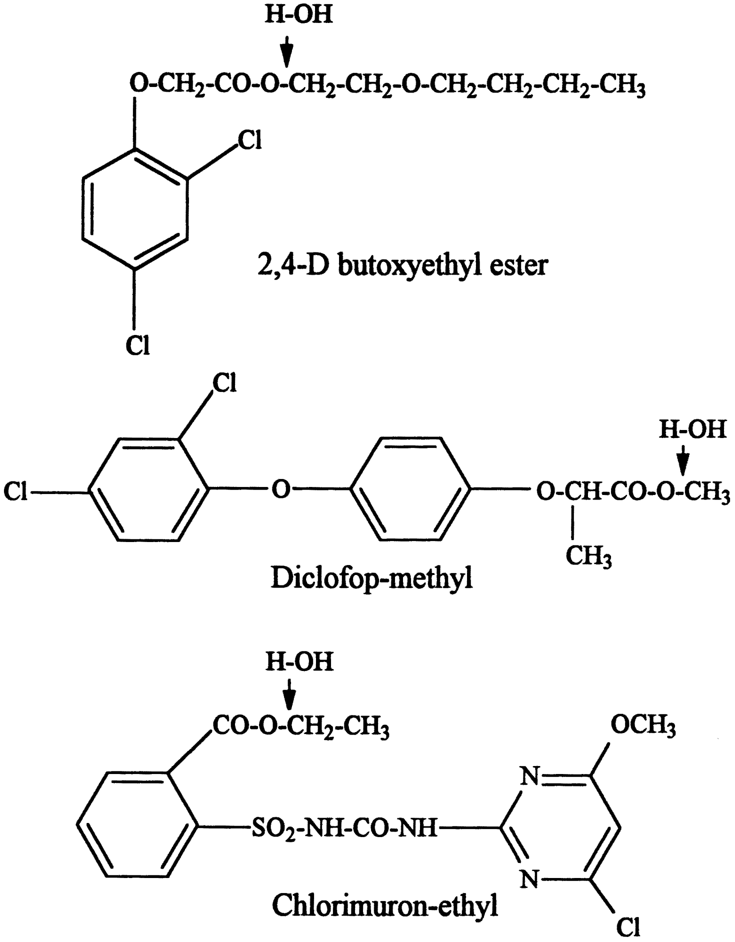

H-OH
O-CH2-CO-O-CH2-CH2-O-CH2-CH2-CH2-CH3
Cl
Cl
2,4-D butoxyethyl ester
Cl
Cl
O
H-OH
O-CH-CO-O-CH3
CH3
Diclofop-methyl
H-OH
CO-O-CH2-CH3
OCH3
N
SO2-NH-CO-NH
N
Chlorimuron-ethyl
Cl

Figure 1. Selected pesticides with susceptibility to esterase-mediated hydrolysis.

acid, is the first enzymatic action on these compounds (*15*). For the PCAs, de-esterification is a bioactivation, not a detoxification mechanism as demonstrated for diclofop-methyl (*5*). The carboxyesterase responsible for de-esterification of the PCA herbicide chlorfenprop-methyl [methyl 2-chloro-3-(4-chlorophenyl)propionate] has been partially purified from oats (*Avena sativa* L.) (*16*) and wild oats (*Avena fatua* L.) (*17*). In tolerant species, the PCA-free acid is detoxified by different mechanisms, i.e., diclofop via arylhydroxylation and subsequent phenolic conjugation (*18*), and fenoxaprop, via glutathione conjugation (*19*). Rice is generally tolerant to fenoxaprop-ethyl, but under low light intensity, rice can be damaged by fenoxaprop-ethyl treatment (*20*). However, similar rates of *in vitro* and *in vivo* fenoxaprop-ethyl de-esterification were found in rice seedlings grown in either, normal light or low light conditions (*21*). Esterase activity was also measured using fluorescein diacetate (FDA) as a substrate (21). In fenoxaprop-ethyl-treated rice, FDA esterase activity was 41 % lower in shaded plants compared to unshaded plants. However, in untreated rice, FDA esterase activity was 22% lower in shaded versus unshaded plants. Hence, the phytotoxic compounds fenoxaprop-ethyl and fenoxaprop acid, persisted longer in shaded plants, which may explain this phytotoxicty to plants under low light conditions.

The herbicide chloramben (3-amino-2,5-dichlorobenzoic acid) was rapidly metabolized to an *N*-glucoside in resistant plant species, while sensitive plants formed the carboxy-glucose ester (*22*). The glucose ester is unstable *in vivo* due to hydrolysis by esterases, thus an equilibrium between the ester and free acid is maintained.

The selectivity of the sulfonylurea herbicides is based on several detoxification pathways, including oxidative and hydrolytic mechanisms (*6*). Soybean (*Glycine max* Merr.), can de-esterify thifensulfuron-methyl {methyl 3-[[[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]amino]sulfonyl]-2-thiophenecarboxylate} to the free acid (non-phytotoxic), but soybean is unable to de-esterify the herbicide analog, metsulfuron-methyl {methyl 2-[[[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)-amino]-carbon-yl]amino]sulfonyl]benzoate} which injures the crop plant. Soybean esterases can hydrolyze thiophene *O*-carboxymethyl esters and *O*-phenylethyl esters, but not *O*-phenyl methyl esters of certain sulfonylureas (*6*). Chlorimuron-ethyl {ethyl 2-[[[[(4-chloro-6-methoxy-2-pyrimidinyl)amino]carbonyl]amino]sulfonyl]-benzoate} detoxificaction occurrs *via* de-esterification of the ethyl group and dechlorination *via* homoglutathione conjugation in soybean (*7*). However, conjugation occurs three-fold more rapidly than de-esterification.

A recent study compared the phytotoxicity of thirteen ester derivatives (C5 to C16) of the herbicide quinclorac (3,7-dichloro-8-quinolinecarboxylic acid) for phytotoxicity against leafy spurge (*Euphorbia esula* L.) (*8*). Foliar application of quinclorac caused rapid death, whereas quinclorac esters applied at higher concentrations to foliage caused only phytotoxicity, not mortality. When applied to soil, quinclorac esters were metabolized by a series of and oxidations while hydrolysis was limited. This resulted in the slow release of quinclorac which increased herbicide efficacy against leafy spurge plants.

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch004

Ester Hydrolysis in Microorganisms

Studies of fenoxaprop-ethyl (*23*, *24*) and diclofop-methyl (*25*) degradation in soils demonstrated a rapid hydrolysis of both compounds to their parent acids. This hydrolysis was more rapid in moist and non-sterile soils compared to dry or sterile soils, which suggested microbial degradation. The role of enzymatic hydrolysis of diclofop-methyl was reduced with certain microbial inhibitors (propylene oxide and sodium azide) (*25*). During hydrolysis of diclofop-methyl and fenoxaprop-ethyl in soil, enantiomeric inversion was observed (*26*). The S enantiomers of both compounds underwent a more rapid inversion than the R enantiomers, and rates of inversion were dependent on soil type. No inversion was observed with entantiomers of the parent compounds. Fenoxaprop acid undergoes further degradation in soil, forming metabolites such as 6-chlorobenoxazolone, 4-[(6-chloro-2-benzoxazolyl)-oxy]phenetole, and 4-[(6-chloro-2-benzoxazolyl)oxy]phenol (*24*). Soil pH also affects the degradation pathway of fenoxaprop-ethyl. Under acidic conditions, the rate of de-esterification was significantly lower than under neutral soil conditions, however, the benzoxazolyl-oxy-phenoxy ether linkage of fenoxaprop-ethyl was prone to non-enzymatic cleavage under acidic conditions (*27*). De-esterification of fenoxaprop-ethyl occurs readily in mixed microbial cultures (*28*) and in pure cultures and enzyme extracts of bacteria, especially fluorescent pseudomonads (*27*, *29*). Fenoxaprop-ethyl de-esterification in bacterial enzyme preparations is pH sensitive, with the highest activity in the neutral to slightly alkaline range. The same *Pseudomonas* strains that hydrolyzed fenoxaprop-ethyl were unable to de-esterify chlorimuron-ethyl (R.M. Zablotowicz, unpublished results). Four distinct types of esterases are found in a *P. fluorescens* strain (*30*). These *P. fluorescens* esterases differ in substrate specificity, cellular location and structure.

Esterases have been cloned, and proteins have been sequenced from several microorganisms, e.g., two ferulic acid esterases from *Aspergillus tubingensis* (*31*), a cephalosporin esterase from the yeast *Rhodosporidium toruloides* (*32*), a chrysanthemic acid esterase from *Arthrobacter globiformis* (*33*), and several other esterases from *Pseudomonas fluorescens* strains (*30*, *34*, *35*). The ability of these esterases to hydrolyze ester linkages of pesticides has not been examined. Since molecular oxygen is not involved, enzymatic hydrolysis can occur under anaerobic and aerobic conditions.

The active site of prokaryotic and eukaryotic esterases contains the serine motif (Gly-X-Ser-X-Gly), originally characterized in serine hydrolase (*36*). This conserved peptide is part of a secondary structure of the enzyme molecule located between a -strand and an α-helix (*37*). A general mechanism has been proposed for the catalytic activity of the esterase superfamily (Figure 2). This mechanism involves the formation two tetrahedral intermediates (*37*), with the active serine serving as a nucleophile enabling ester bond cleavage. A histidine residue in a β-strand, is also involved in formation of an ionic attachment at the carbonyl oxygen atom of the substrate during catalysis. Reaction of the second intermediate, with a molecule of water, concomitantly releases the parent acid of the substrate and regenerates the active serine of the enzyme. A similar reaction mechanism is postulated for the serine

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch004

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch004

proteases. However, a chrysanthemic acid esterase from *Arthrobacter* sp. is similar to many bacterial amidases that possess the Ser-X-X-Lys motif in the active site. This esterase may provide a unique opportunitiy for biotechnological synthesis, since it stereoselectively produces (+)-*t*-chrysanthemic acid, used in pyrethroid insecticide synthesis.

Certain esterases, e.g., the *R. toruloides* cephalosporin esterase, also have acetylating activity when suitable acetyl donors are present (*32*). The *R. toruloides* cephalosporin esterase is a glycoprotein (80 kDa glycosylated; 60-66 kDa de-glycosylated) with eight potential binding sites (Asn-X-Ser or Asn-X-Thr) for glucose. When de-glycosylated, enzyme activity was reduced about 50%, indicating an important role for glycosylation in stabilizing the native protein structure.

Feng *et al.* (*38*) transformed tobacco (*Nicotiana tabacum* L.) and tomato (*Lycopersicon esculentum* L.) with genes encoding for rabbit liver esterase 3 (RLE3). This esterase gene expressed in these plants, conferred resistance to the herbicide thiazopyr [methyl 2-(difluoromethyl)-5-(4,5-dihydro-2-thiazolyl)-4-(2-methylpropyl)-6-(trifluoromethyl)-3-pyridinecarboxylate]. These researchers proposed, that a critical assessment of microbial esterases may provide other hydrolytic enzymes that are useful in conferring pesticide (herbicide) resistance in plants. Such enzymes could also play a role in reducing the levels of pesticides, their metabolites, and other potentially harmful xenobiotics in food products.

Inhibition of Esterases in Plants and Microorganisms

The effects of two fungicides, captan [*N*-(trichloromethylthio)-4-cyclohexene-1,1-dicarboximide] and folpet [*N*-(trichloromethylthio)phthalimide], and a sufhydryl binding inhibitor, perchloromethylmercaptan, were assessed on esterase activity of *Penicillium duponti* (*39*). Esterase activity using *p*-nitrophenylpropionate as substrate was inhibited 50% by all three compounds at 0.5 to 2.0 μM. But, concentrations that totally inhibited *p*-nitrophenylpropionate esterase activity had no effect on α-naphthyl acetate esterase activity. The extreme sensitivity of certain fungal esterases to these two fungicides, suggests that part of their toxicity to fungi may be due to esterase inhibition (*39*).

Amide Hydrolysis in Plants

Amide and substituted amide bonds are present in several classes of pesticides, i.e., acylanilides: alachlor [2-chloro-*N*-(2,6-diethylphenyl)-*N*-(methoxymethyl)acetamide]; carboxin (5,6-dihydro-2-methyl-*N*-phenyl-1,4-oxathiin-3-carboxamide); diphenamid {2-chloro-*N*-[(1-methyl-2-methoxy)ethyl]-*N*-(2,4-dimethyl-thien-3-yl}; metalaxyl [*N*-(2,6-dimethylphenyl)-*N*-(methoxyacetyl)-alanine methyl ester]; metolachlor [2-chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-(2-methoxy-1-methylethyl)acetamide]; and propanil, [*N*-(3,4-dichlorophenyl)propionamide], phenylureas: diuron [*N*-(3,4-dichlorophenyl)-

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch004

N,*N*-dimethylurea]; fluometuron {*N*,*N*-dimethyl-*N*-[3-(trifluoromethyl)phenyl]urea}; and linuron, [*N*-(3,4-dichlorophenyl)-*N*-methoxy-*N*-methylurea]; and carbamates: IPC (isopropyl carbanilate); and CIPC (isopropyl *m*-chlorocarbanilate). Some structural examples of these substituted amides are given for comparison (Figure 3.)

Rice (*Oryza sativa* L.) is tolerant to the acylanilide herbicide propanil, due to the presence of high levels of aryl acylamidase (EC 3.5.1.a), which hydrolyzes the amide bond to form 3,4-dichloroaniline (DCA) and propionic acid (*40*, *41*). This enzyme activity is the biochemical basis of propanil selectivity in the control of barnyardgrass [*Echinochloa crus-galli* (L.) Beauv.] in rice. Barnyardgrass tissue was unable to detoxify absorbed propanil due to very low enzymatic activity; rice leaves contained sixty-fold higher aryl acylamidase activity than barnyardgrass leaves (*40*). Propanil aryl acylamidase activity is also widely distributed in other crop plants and weeds (*42*, *43*). Plant aryl acylamidases have been isolated, and partially purified and characterized from tulip (*Tulipa gesnariana* v.c. Darwin) (*44*), dandelion (*Taraxacum officinale* Weber) (*45*), and the weed red rice (*Oryza sativa* L.) (*46*). Red rice is a serious conspecific weed pest in cultivated rice fields in the southern U.S. (*47*), and its ability to hydrolyze propanil limits the utility of this herbicide where red rice is present. Propanil hydrolysis by aryl acylamidases has also been observed in certain wild rice (*Oryza*) species (*48*).

With intensive use of propanil in Arkansas rice production over a thirty-five year period, barnyardgrass (initially controlled by propanil), has evolved resistance to this herbicide, and this biotype is currently a serious problem (*49*). Populations of propanil-resistant barnyardgrass have been verified in all southern U.S. states that use propanil in rice cultivation. A series of experiments with propanil-resistant barnyardgrass showed that the mechanism of resistance was increased propanil metabolism by aryl acylamidase activity (*50*, *51*). Increased propanil metabolism by aryl acylamidase was also shown to be the resistance mechanism in another related weed, junglerice [*Echinochloa colona* (L.) Link] (*52*).

Naproanilide [2-(2-naphthyloxy)-propionanilide], a herbicidal analog of propanil, was hydrolyzed by rice aryl acylamidase (*53*). The cleavage product, napthoxypropionic acid is phytotoxic, however, it is hydroxylated and subsequently glucosylated as a detoxification mechanism in rice. Naproanilide hydrolysis also occurred in a susceptible plant (*Sagittaria pygmaea* Miq.), but napthoxypropionic acid was not metabolized further in this species (53).

Initial assessment of substrate chemical structure and aryl acylamidase activity has been studied in enzyme preparations from various plants, e.g., rice (*40*), tulip (*44*), dandelion (*45*), and red rice (*46*). Nine mono- and dichloro-analogs of propanil were examined for substrate specificity of these enzyme preparations. Different profiles of hydrolytic rates were observed among species. In all species tested (not tested in red rice), little or no activity was observed with 2,6-dichloropropionanilide. In rice, greater activity was observed with 2,3-dichloropropionanilide compared to propanil; while in tulip, equal activity was observed with propanil, 2,4-dichloropropionanilide, and 4-chloropropionanilide as substrates. In a similar fashion, the effects of alkyl chain length on 3,4-dichloroanilide substrates were evaluated. Propanil was the best substrate for all four enzyme preparations. Reducing the chain length to one carbon

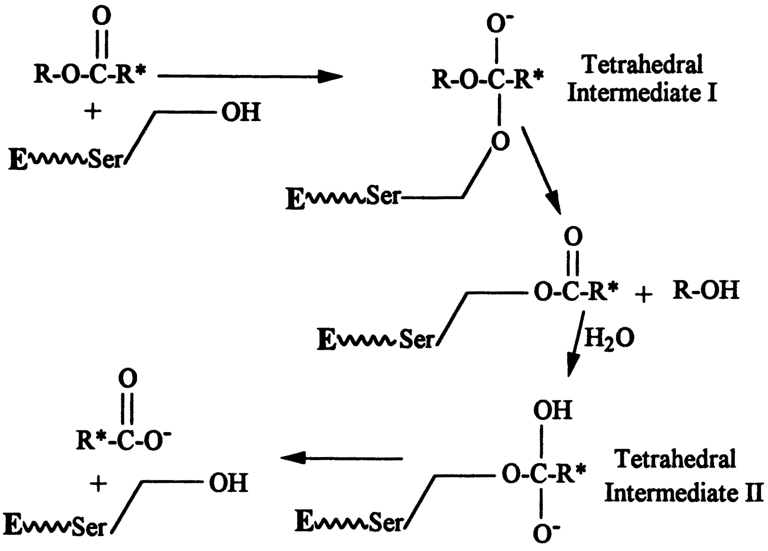

R-O-C-R*
E∿∿∿Ser
OH
Tetrahedral Intermediate I
R-O-C-R*
+ R-OH
H2O
Tetrahedral Intermediate II
R*-C-O-

Figure 2. Schematic mechanism of esterase-mediated hydrolysis.

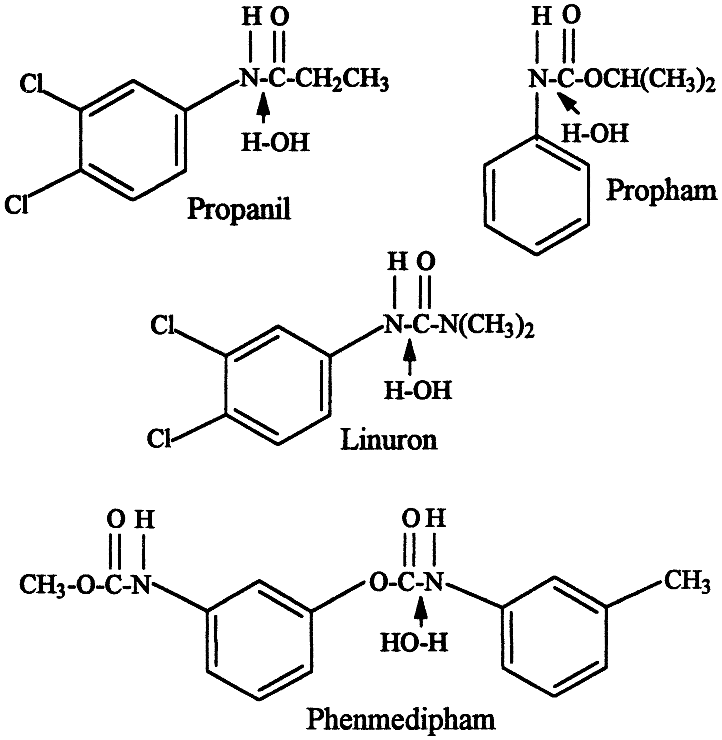

N-C-CH2CH3
H-OH
Cl
Propanil
N-C-OCH(CH3)2
H-OH
Propham
N-C-N(CH3)2
H-OH
Linuron
CH3-O-C-N
O-C-N
HO-H
CH3
Phenmedipham

Figure 3. Selected pesticides with amide and substituted amide bonds.

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch004

(i.e., the acetamide analog), decreased activity by 18 to 50%, compared to propanil. Increasing the alkyl chain length or inserting alkyl branching, reduced activity by 60 to 100%.

Aryl acylamidases from several plant species have been purified to homogeneity (*54*). The enzymes from orchardgrass and rice are quite similar. Both have molecular weights of about 150 kDa, and are membrane bound. These enzymes share a common pH optimum of 7.0, similar Km's for propanil, and are both inhibited by the insecticide carbaryl. A gene for aryl acylamidase has been cloned from Monterey pine (*Pinus radiata*) male pine cones (*55*). This protein, comprised of 319 amino acids, is similar to esterases containing a serine hydrolase motif in the active site.

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch004

Amide Hydrolysis in Microorganisms

As we have reviewed (*56*), aryl acylamidases have been characterized in diverse species of algae, bacteria and fungi, and propanil has been the most studied pesticide substrate. The distribution of aryl acylamidases and related hydrolytic enzymes produced by selected microorganisms are summarized (Table II). Differences in substrate specificity and inducibility of these enzymes are found among genera and species of microbes. For example, aryl acylamidases produced by *P. fluorescens* strains RA2 and RB4 had a substrate range limited to certain acylanilides (propanil, nitroacetanilide, and acetanilide), and were ineffective on several herbicides containing substituted amide bonds: phenylureas (linuron and diuron), a phenylcarbamate (CIPC), chloracetamides [alachlor and the *N*-dealkylated metabolite 2-chloro-*N*-(2',6'-diethylacetanilide], and a benzamide {pronamide [3,5-dichloro(*N*-1,1-dimethyl-2-propynyl)benzamide]} (*66*). Similar substrate specificities are found in other bacterial strains (Table III). Other microbial acylamidases also have activity restricted to certain acylanilides, e.g., *Fusarium oxysporum* (*69*).

Some acylamidases, such as a linuron-inducible enzyme produced by *Bacillus sphaericus* (*59*), and an extracellular coryneform aryl acylamidase (*60*), have wide substrate specificities including hydrolytic action on acylanilide, phenylcarbamate, and substituted phenylurea pesticides. In a *Fusarium oxysporum* strain, there are two distinct aryl acylamidases: one induced by propanil, the second induced by *p*-chlorophenyl methyl carbamate (*69*). One study has compared the metabolism of several phenylurea herbicides by bacteria and fungi (*70*). Nine fungal species were more effective in degrading linuron or isoproturon [3-(4-isopropylphenyl)-1,1-dimethylurea] compared to fenuron (1,1-dimethyl-*N*-phenylurea), but *N* or *O*-dealkylation (not hydrolysis) was the major degradation mechanism. Only one of five bacterial isolates (a pseudomonad) metabolized linuron with the formation of 3,4-dichloroaniline, indicating hydrolytic cleavage of the urea bond. The hydrolytic mechanism for phenylurea herbicides by a coryneform-like bacteria has also been shown with an organism capable of hydrolyzing linuron > diuron > monolinuron [*N*-(3-chlorophenyl)-*N*-methoxy-*N*-methylurea] >> metoxuron [*N*-(3-chloro-4-methoxyphenyl)-*N*,*N*-dimethylurea] >>> isoproturon (*71*).

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch004

Table II. Selected Microbial Species that Produce Aryl Acylamidases and Related Hydrolytic Enzymes

Organism	*Substrate*	*Inducer*	*Citation*
Anacystis nidulans	CIPC, IPC	nt*	(*57*)
Aspergillus nidulans	Propanil, (propionanilide**)	Constitutive	(*58*)
Bacillus sphaericus	Linuron, carboxin, IPC	Linuron	(*59*)
Coryneform-like, strain A-1	CIPC, linuron, naproanilide, propanil	(Acetanilide)	(*60*)
Fusarium oxysporum	Propanil, CIPC, (acetanilide)	Propanil and phenylureas	(*61*)
Fusarium solani	Propanil, (acetanilide)	Propanil and (acetanilide)	(*62*)
Nostoc entophytum	Propanil	nt	(*63*)
Penicillium sp.	Karsil, propanil, (acetanilide)	Karsil	(*64*)
Pseudomonas fluorescens	(Acetanilide), (*p*-Nitroacetanilide, *p*-Hydroxyacetanilide)	(Acetanilide)	(*65*)
P. fluorescens	Propanil, (acetanilide, nitroacetanilide),	Constitutive	(*66*)
P. picketti	Propanil	Constitutive	(*67*)
P. striata	CIPC, IPC, Propanil	CIPC	(*68*)

NOTE: *nt = not tested; ** compounds in parenthesis are not used as pesticides.

Propanil hydrolysis yielding DCA (*72,73*) is the major mechanism for dissipation of this compound in soil. Many microorganisms hydrolyze propanil to DCA, and enzymes from several species have been isolated, partially purified, and characterized. For example, ninety seven bacterial isolates were collected from soil and flood water of a Mississippi Delta rice field over a two year period following propanil application (Table IV). Overall, 37% of the soil and water isolates exhibited propanil hydrolytic activity. Although activity was observed in both gram-positive and gram-negative isolates, there was a greater frequency of propanil-hydrolysis among gram-negative bacteria. The hydrolytic activity of cell-free extracts of several of these isolates, and other rhizobacterial cultures on several substrates, was assessed using methods described elsewhere (*66*). Only acylanilides were hydrolyzed, with no detectable hydrolysis of carbamate and substituted urea herbicides (Table V). All isolates, except AMMD and UA5-40, were isolated from soil or water that had been previously

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch004

Table III. Aryl Acylamidase Activity on Five Substrates in Cell-Free Extracts from Ten Bacterial Strains

Genera	*Strain*	*Source*	*Product formed (nmol mg^{-1} protein h^{-1})*				
			Propanil	*2-NAA*	*Acetanilide*	*Linuron*	*CIPC*
Bacillus sp.	S92B2	S	18.1	4.6	16.4	nd	nd
Flavobacterium sp.	W92B14	W	59.2	38.7	28.2	nd	nd
P. cepacia	AMMD	R	13.9	0.6	5.1	nd	nd
P. fluorescens	RA-2	R	14,310	5,540	15,940	nd	nd
P. fluorescens	RB-3	R	14.0	254	49	nd	nd
P. fluorescens	RB-4	R	35,218	11,017	30,510	nd	nd
P. fluorescens	UA5-40	R	0.3	1.4	1.4	nd	nd
P. fluorescens	W92B12	W	14.5	25.8	17.0	nd	nd
Rhodococcus sp.	S92A1	S	15.9	10.2	3.5	nd	nd
Rhodococcus sp.	S93A2	S	144	18.7	191	nd	nd

NOTE: S = soil isolate; W= water isolate; R = rhizosphere isolate. Cell-free extracts (CFE, 1 to 8 mg protein mL) were prepared as described elsewhere (*66*). The assay mixture contained 800 nmol substrate and 0.2 mL CFE in a final volume of 1.0 mL potassium phosphate buffer (pH 8.0, 50 mM), incubated at 30°C for 15 min to 4 h. Acetanilide hydrolysis determined by diazotization; 2-NAA (2-nitroacetanilide) hydrolysis determined spectrophotometrically at 410 nm (66); Propanil, CIPC, and linuron hydrolysis determined by HPLC (*66*).

Table IV. Recovery of Propanil-Hydrolyzing Isolates from a Mississippi Delta Rice Soil and Flood Water

Year	*Source*	*Gram-stain reaction*	*Propanil-hydrolyzing isolates*	*Total Isolates tested*
1982	Soil	Negative	9	13
		Positive	2	19
	Water	Negative	4	8
		Positive	6	15
1983	Soil	Negative	5	9
		Positive	3	11
	Water	Negative	3	8
		Positive	4	14
	Total soil		19	52
	Total, water		17	45
	Total gram-positive		15	59
	Total gram-negative		21	38

NOTE: Bacteria were isolated on tryptic soy agar (10%), from soil and water following propanil application. Individual colonies were subcultured, ascertained for purity and tested for Gram stain reaction. Propanil hydrolysis assessed in cell suspensions (log 11.0 cells ml; 50 mM potassium phosphate, pH 8.0; 800 µM propanil) after 24 h incubation. Propanil and metabolites were determined by HPLC as described elsewhere (*66*).

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch004

exposed to propanil. *P. fluorescens* strains RA-2 and RB-4 exhibited aryl acylamidase activity several orders of magnitude higher than any other organism isolated in our studies (*56, 66*).

The ability to hydrolyze propanil was studied in fifty-four isolates of fluorescent pseudomonads collected from three Mississippi Delta lakes (*74*). Overall, about 60% of the isolates hydrolyzed propanil, and all the propanil-hydrolyzing bacteria were identified as *P. fluorescens* biotype II. Most propanil-hydrolyzing *P. fluorescens* isolates transformed DCA to 3,4-dichloroacetanilide. The potential for acetyl transferase activity by these *P. fluorescens* aryl acylamidases should not be overlooked, since the enzyme isolated from *Nocardia globerula* (*75*) and *Pseudomonas acidovarans* (*76*) possesses this activity. The amidase from *Rhodococcus* sp. strain R312 has both amidase and acyl transferase activity. This enzyme was expressed in *Escherichia coli* and utilized in kinetic studies on acyl transferase activity (*77*). This purified enzyme catalyzed acyl transfer from amides and hydroxamic acids to only water or hydroxylamine. Further aspects of acetyl transferase activity will be addressed in the later presentation on bialaphos and phosphinothricin detoxification/resistance.

A variety of methods (radiological, spectrophotometric, and HPLC) are available to measure enzymatic hydrolysis of amides. A colorimetric method for measuring aryl acylamidase activity in soil using 2-nitroacetanilide (2-NAA) as substrate was recently developed (*78*). Aryl acylamidase (2-NAA) activity was several-fold lower than other

Table V. ^{14}C-Propanil Metabolism by Cell Suspensions of Soil, Water and Rhizosphere Bacteria

Genera	*Strain*	*Source*	*% ^{14}C Recovered in methanolic extracts*			
			Propanil	*DCA*	*3,4-DCAA*	*Origin*
Bacillus sp.	S92B2	S	nd	71.1	10.5	18.5
Flavobacterium sp.	W92B14	W	nd	88.6	4.5	6.9
P. cepacia	AMMD	R	74.5	6.9	18.6	Nd
P. fluorescens	RA-2	R	nd	100	Nd	Nd
P. fluorescens	RB-3	R	2.5	91.0	5.0	1.5
P. fluorescens	RB-4	R	nd	100	Nd	Nd
P. fluorescens	UA5-40	R	100	nd	Nd	Nd
P. fluorescens	W92B12	W	70.3	9.2	4.2	16.3
Rhodococcus sp.	S92A1	S	15.9	10.2	3.5	Nd
Rhodococcus sp.	S93A2	S	nd	96.1	3.9	Nd

NOTE: DCA = 3,4-dichloroaniline; 3,4-DCAA = 3,4-dichloroacetanilide; Origin = highly polar metabolites (immobile in benzene: acetone solvent); S = soil isolate; W= water isolate; R = rhizosphere isolate; nd = none detected. Cell suspensions [48 h tryptic soy broth cultures, log 11.0 cells ml^{-1}; potassium phosphate buffer (50 mM, pH 8.0)] were treated with propanil (800 μM, 8.0 kBq mL^{-1}) and incubated at 28°C, 150 rpm, 24 h. Propanil and metabolites identified by TLC and radiological scanning as described elsewhere (*56*).

hydrolytic enzymes such as alkaline phosphatase (3 to 5%), and aryl sulfatase (5 to 13%). Aryl acylamidase activity was 1.3- to 2.5-fold higher in surface no-till soils compared to conventional-tilled soils. This would be expected due to the greater microbial populations and diversity associated with the accumulation of soil organic matter in the surface of no-till soils. Maximal aryl acylamidase activity was observed at pH 7.0 to 8.0, and activity was reduced at assay temperatures above 31°C. Thermal inactivation has also been reported for a purified aryl acylamidase from *P. fluorescens* (*65*).

With respect to chloroacetamide herbicides, there is only limited evidence in the literature for cleavage of the substituted amide bond. The fungus *Chaetomium globosum* was shown to hydrolyze substituted amide bonds of alachlor (*79*) and metolachlor (*80*). A unique cleavage of propachlor [2-chloro-*N*-(1-methylethyl)-*N*-phenylacetamide] at the benzyl C-N bond, by a *Moraxella* isolate, has been demonstrated (*81*). However, little is known about this mechanism, or its distribution among other species. Recently, two bacteria (*Pseudomonas* and *Acientobacter*) capable of metabolizing propachlor were described (*82*). Both strains initially dehalogenated propachlor to *N*-isopropylacetanilide. The *Acientobacter* strain then hydrolyzed the amide bond, before the release of isopropylamine, and prior to ring cleavage. The *Pseudomonas* strain initially transformed propachlor via *N*-

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch004

dealkylation. This metabolite was then cleaved at the benzyl C-N bond as in the *Moraxella* isolate described above (*81*).

Aryl acylamidases have been purified from several bacterial species including, *B. sphaericus* (*59*), a coryneform-like bacterium (*60*), *Nocardia globerula* (*75*), *P. aeuriginosa* (*83*), *P. fluorescens* (*65*), and *P. pickettii* (*67*). These various enzymes have been shown to be quite diverse. Among these four genera, the aryl acylamidases ranged in size from 52.5 kDa for the *P. fluorescens* enzyme, to about 127 kDa for the coryneform and *N. globerula* aryl acylamidases. The *P. pickettii* enzyme is a homodimer, while the other enzymes are monomers. A novel amidase from *P. putida*, specific for hydrolyzing *N*-acetyl arylalkylamines, was purified to homogeneity (*84*). This protein (MW =150 kDa) is a tetramer of four identical subunits. This enzyme hydrolyzed various *N*-acetyl arylalkylamines containing a benzene or indole ring, and acetic acid arylalkyl esters, but not acetanilide derivatives. To our knowledge, no genes for specific pesticide-hydrolyzing aryl acylamidases have been cloned. A multiple alignment and cluster analysis has been performed on amino acid sequences of 21 amidases or amidohydrolases (*85*). A hydrophobic conserved motif [Gly-Gly-Ser-Ser (amidase signature)] has been identified which may be important in binding and catalysis. Amidases from prokaryotic organisms also have a conserved C-terminal end, not found in eukaryotes. These studies also indicate similarities of amino acid sequences among amidases, nitrilases and ureases.

Metals, i.e., Hg^{++}, Cu^{++} $Cd^{++}$and Ag^{+}, that affect sulfhydryl groups differentially inhibited bacterial aryl acylamidases (*59, 60, 65, 83*). EDTA did not inhibit the *B. sphaericus* (*59*) and *P. fluorescens* (*65*) enzymes, but 2.5 mM EDTA inhibited the coryneform-like aryl acylamidase by about 90% (*60*). An aryl acylamidase from *P. aeuriginosa* required Mn^{++} or Mg^{++} (*83*), but these cations were not required by other enzymes mentioned above.

Initial studies of the interaction of chemical structure and microbial (*Penicillium* sp.) aryl acylamidase activity were evaluated in an enzyme inducible by karsil [*N*-(3,4-dichlorophenyl)-2-methylpentanamide] (*64*). Activity was greater with longer alkyl amide substitution, i.e., activity was 4, 262 and 1000 units for acetanilide, propionanilide, and butryanilide, respectively. Of the six herbicides evaluated, propanil had the highest activity (520 units), compared to karsil (70 units), solan [*N*-(3-chloro-4-methylphenyl)-2-methylpentanamide] (55 units), dicryl [*N*-(3,4-dichlorophenyl)-2-methyl-2-propenamide] (40 units), and no activity was detected with diuron and CIPC. The hydrolysis of seven *para*-substituted acetanilides by three bacterial species (*Arthrobacter* sp., *Bacillus* sp., and *Pseudomonas* sp.) was compared to rate constants for alkaline-mediated hydrolysis (*86*). In these studies, *para* substitution did not affect acetanilide hydrolysis by resting cells of these bacteria, however alkaline-mediated hydrolysis was highly affected. These studies suggest different intermediates and mechanisms for biotic versus abiotic transformations.

A structure-activity study evaluated acylanilide herbicide chemical structure using model substrates and four bacterial strains [two *Arthrobacter* spp. (BCL and MAB2), a *Corynebacterium* sp. (DAK12), and an *Acinetobacter* sp. (DV1)] capable of growth on acetanilide (*87*). When the nitrogen of acetanilide was alkylated (methyl or ethyl), the compound was an unsuitable substrate for all four strains. When a *para*-

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch004

methyl group was present on the acetanilide ring, activity occurred in all strains, but was lower than acetanilide activity in DV1 and one of the *Arthrobacter* strains, (MAB2). When the methyl group was in the *ortho* or *meta* position, activity similar to that on acetanilide occurred in two strains (DAK12 and BCL), but was only about 30% of the acetanilide rate in MAB2, and undetectable in DV1. Little or no activity in all four strains was found with dimethyl substitution in the 2 and 6 positions. Consequently, alachlor and metolachlor (with 2 and 6 alkyl substitutents on the ring) are more persistent in soil compared to propachlor with an unsubstituted aniline ring (*88*).

The fungicide ipridione [3-(3,5-dichlorophenyl)-*N*-isopropyl-2,4-dioxoimidazolidine-1-carboxamide] undergoes several potential amide hydrolytic reactions. Ipridione degradation by an *Arthrobacter*-like strain (*89*) and three pseudomonads (*P. fluorescens*, *P. paucimobilis*, and *Pseudomonas sp.*) has been reported (*90*). Initial cleavage of ipridione forms *N*-(3,5-dichlorophenyl)-2,4-dioximidazoline and isopropylamine. The imidazolidine ring is cleaved, forming (3,5-dichlorophenylurea)acetic acid, which can be further hydrolyzed to 3,5-dichloroaniline. 3,5-Dichloroaniline is a major metabolite observed in soils where microflora have adapted the ability for enhanced ipridione degradation (*91*).

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch004

Inhibition of Plant and Microbial Amidases

Propanil hydrolysis is inhibited by various carbamate and organophosphate insecticides in plants (*40*, *92*) and microorganisms (*93*). Competitive inhibition of aryl acylamidase activity by these compounds was the basis for increased (synergistic) injury to rice, caused when insecticides were applied to rice in close proximity to propanil application (*94*). Synergistic effects of propanil with several agrochemicals: carbaryl (1-naphthyl *N*-methylcarbamate); anilofos {*S*-[2-[(4-chlorophenyl)(1-methylethyl)-amino]-2-oxoethyl] *O*,*O*-dimethyl-phosphoro-dithioate}; pendimethalin [*N*-(1-ethylpropyl)-3,-dimethyl-2,6-dinitrobenzenamine]; and piperophos {*S*-[2-(2-methyl-1-piperidinyl)-2-oxoethyl]*O*,*O*-dipropyl phosphorodithioate} in propanil-resistant barnyardgrass were recently detected using a chlorophyll fluorescence technique (*95*). Carbaryl's synergism is due to its competitive inhibition of aryl acylamidase (*40*), but the exact mechanism of the other synergists in propanil-resistant barnyardgrass is presently unknown.

Interactions of insecticides and soil aryl acylamidase activity have also been reported (*93*). When soil was treated with *p*-chlorophenyl methyl carbamate, propanil hydrolysis was substantially inhibited, and subsequent formation of tetrachlorodiazobenzene was reduced 10- to 100-fold. Carbaryl at 100 μM inhibited propanil aryl acylamidase activity by 10 to 70% in several bacterial strains (*66*, *96*). The *Fusarium solani* propanil-aryl acylamidases were insensitive to high concentrations of carbaryl and parathion (*O*,*O*-diethyl-*O*-4-nitrophenyl phosphorothioate), however the chloroacetanilide, herbicide ramrod (*N*-isopropyl-2-chloroacetanilide), competitively inhibited acetanilide hydrolysis.

Carbamate Hydrolysis in Plants

Carbamates have a broad spectrum of pesticidal activity and include commonly used insecticides, herbicides, nematicides (aldicarb {2-methyl-2-(methylthio)propanal *O*-[(methylamino)cabonyl]oxime} and fungicides. There are three major classes of carbamates: methyl carbamates: aldicarb, carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranol methylcarbamate), and carbaryl; phenylcarbamates: CIPC, IPC, and phenmedipham; and thiocarbamates: EPTC (*S*-ethyl dipropyl carbamothioate), butylate [*S*-ethyl bis(2-methylpropyl)carbamothioate], and vernolate (*S*-propyl dipropylcarbamothioate). The behavior of insecticidal carbamates has been extensively reviewed (*97*, *98*). Studies on IPC (*99*) and CIPC (*100*, *101*) in plants indicate that the major metabolic route is aryl hydroxylation and conjugation. Plants do not cleave the carbamate bond of the phenylcarbamate herbicides, which is distinct from the initial metabolism in either microorganisms or animals. The thiocarbamates such as EPTC are metabolized in tolerant species such as corn, cotton (*Gossypium hirsutum*), and soybeans via initial oxidation to the sulfoxide followed by glutathione conjugation (*102*, *103*). The pathway for EPTC metabolism in plants is similar to that found in mouse liver microsomes (*104*).

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch004

Carbamate Hydrolysis in Microorganisms

Microbial degradation of carbamates occurs readily in soil. Accelerated degradation of certain carbamate insecticides has led to ineffectiveness of these compounds, e.g., control of phyloxera in vineyards by carbofuran (*105*). Bacterial isolates from several genera (*Arthrobacter*, *Achromobacter*, *Azospirillum*, *Bacillus*, and *Pseudomonas*) can hydrolyze various insecticidal carbamates (*106*). Hydrolysis is the major pathway for the initial breakdown of carbofuran, but little is known about the fate of the metabolites formed by this mechanism (*106*). Mineralization of the carbonyl group of carbofuran occurs more extensively compared to mineralization of the ring structure (*107*). The toxicity of aldicarb is greatly reduced when it is hydrolyzed to the oxime and nitrile derivatives (*108*), but oxidation of aldicarb to the sulfone or sulfoxide yields compounds with similar or greater toxicity. Organophosphorus compounds: paraoxon (phosphoric acid diethyl-4-nitrophenyl ester), chlorfenvinphos [phosphoric acid 2-chloro-1-(2,-4-dichlorophenyl)ethyl diethyl ester], and disulfoton {phosphorodithioic acid *O*,*O*-diethyl *S*-[2-(ethylthio)ethyl]ester} which are esterase inhibitors, suppressed carbofuran hydrolysis in soil for 3 to 21 days (*109*). The persistence of carbofuran was increased when these esterase inhibitors were combined with the cytochrome P-450 inhibitor, piperonyl butoxide. This synergism is due to the inhibition of both major mechanisms of carbofuran degradation, i.e., hydrolysis and oxidation.

Some microbial aryl acylamidases [*P. striata* (*110*), *B. sphaericus* (*59*) and a coryneform-like isolate (*60*)] can hydrolyze certain phenylcarbamates, e.g., CIPC and IPC, as summarized in Table II. However, the *P. striata* aryl acylamidase is unable to hydrolyze methylcarbamates such as carbaryl. Carbamate hydrolases such as the

Arthrobacter phenmedipham-hydrolase, is specific for phenylcarbamates (*111*), whereas the *Achromobacter* carbofuran-hydrolase is specific for methylcarbamates (*112*).

A cytosolic carbamate-hydrolase has been purified from a *Pseudomonas* sp. (*113*). This enzyme is composed of two identical dimers with a molecular weight of 85 kDa each, and is active on carbaryl, carbofuran and aldicarb as substrates (*113*). The *Achromobacter* carbofuran-hydrolase has been purified to homogeneity and has a molecular weight of 150 kDa. This enzyme is either cytoplasmic or occurs in the periplasmic space, and requires Mn^{++} as an activator (*114*). It has no urease activity and does not hydrolyze benzamide. The genes for the *Achromobacte*r carbofuran-hydrolase have been cloned, however they were poorly expressed in many gram-negative bacteria such as *E. coli*, *Alcaligenes eutrophus,* and *P. putida.* This indicates that secondary processing is required to produce a functional enzyme. The genes for phenmedipham-hydrolase (*pcd*) have also been cloned and the protein purified to homogeneity (*111*). The phenmedipham-hydrolase is a monomer with a molecular weight of 55 kDa, and contains the esterase motif (Gly-X-Ser-X-Gly). The phenmedipham-hydrolase also has hydrolytic activity on the traditional esterase substrate, *p*-nitrophenylbutyrate. The *pcd* gene was expressed in tobacco, and conferred resistance to phenmedipham at rates 10-fold higher than normal field application rates (*115*). Although bacteria have been isolated that possess diverse hydrolytic degradation mechanisms for carbaryl, few organisms are capable of complete mineralization of the entire molecule. When a bacterial consortium (two *Pseudomonas* spp.) was constructed, both hydrolysis and aromatic mineralization of carbaryl occurred (*116*).

Thiocarbamates have been developed as herbicides (butylate, EPTC and vernolate) and fungicides. Repeated application of these compounds to soil led to the development of microbial populations with accelerated thiocarbamate degradation capability. Soils adapted to EPTC degradation also rapidly degraded a related thiocarbamate, vernolate (*117*). However, soils adapted to butylate did not rapidly degrade EPTC and vernolate (*117*). EPTC metabolism occurrs in many genera of bacteria (*Arthobacter*, *Bacillus*, *Flavobacterium*, *Pseudomonas*, and *Rhodococcus*) and fungi (*Fusarium*, *Paecilomyces*, *Penicillium*) (*118*). Both hydrolytic and oxidative mechanisms have been proposed for EPTC degradation due to the fact that dipropropylamine was found in the media of *Arthrobacter* and *Rhodococcus* strains TE1 (*119*) and BE1 (*120*). Evidence from another *Rhodoccoccus* strain JE1, indicates initial hydroxylation of the propyl group of EPTC, followed by *N*-dealkylation, forming propionaldehyde and *N*-depropyl-EPTC (*121*). Cytochrome P-450s, responsible for EPTC *N*-dealkylation, have been cloned from several *Rhodococcus* strains (*122-124*).

Hydrolysis of Organophosphate Insecticides by Plants

Organophosphate insecticides {parathion, malathion [*S*-1,2-bis(carbethoxy)ethyl-*O*-*O*-dimethyl dithiophosphate], and coumaphos (*O*-3-chloro-4-methyl-2-oxo-2H-

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch004

chromen-7-yl *O,O*-diethyl phosphorothioate)} have replaced many of the chlorinated insecticides, because they are more effective and less persistent. Wheat (*Triticum aestivum* L.) and sorghum (*Sorghum vulgare* L.) rapidly degrade dimethoate [*O,O*-deimethyl-*S*-(*N*-methylcarbamoylmethyl)phosphorothiolothionate] to products, which suggests hydrolytic metabolism (*125*). Crude enzyme extracts from wheat germ were able to hydrolyze malathion to dimethylphosphorothionate and dimethylphosphorothiolthionate (*126*). Metabolism of *O*-ethyl *O*-(4-methylthio)phenyl *S*-propylphosphorodithioate (sulprofos) was studied in cotton (*127*). The major metabolites were the sulfoxide and sulfone derivatives, indicating oxidation as a major initial transformation. Formation of the phenol and gluocoside conjugates of sulprofos indicates hydrolysis of the phospho-phenol bond, but enzymatic hydrolysis has not been confirmed. However, other enzymes, such as mixed-function oxidases and glutathione S-transferases (GST), may be equally important in the detoxification of organophosphorus insecticides in plants (*128*).

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch004

Hydrolysis of Organophosphate Insecticides by Microorganisms

The degradation of organophosphate insecticides has been studied extensively in several gram-negative bacterial strains, especially *Pseudomonas diminuta* and in *Flavobacterium* ATCC 27551 (*129*). Hydrolysis of the organophosphorus insecticides occurs via nucleophilic addition of water across the acid anhydride bond; thus the enzymes named parathion-hydrolases and phosphotriesterases are actually organophosphorus acid anhydrases (*130*). The parathion-hydrolase can be either cytosolic or membrane-bound, depending upon the bacterial species. The *Flavobacterium* enzyme is membrane-bound, and is a single unit of 35 kDa, whereas the enzyme from strain SC (gram-negative, oxidase-negative aerobic, non-motile, rod shaped bacterium) is composed of four identical subunits, each with a molecular weight of 67 kDa (*129*). This enzyme is also a membrane-bound protein (*129*). A coumaphos-degrading *Nocardia* isolate, B-1 (*131*) produces a cytoplasmic parathion hydrolase composed of a single 43 kDa subunit (*129*). These results indicate that enzymes possessing very diverse characteristics can have the same catalytic function. The aryldiphosphatase gene from *Nocardia* strain B-1 (*adp*-gene) has nothing in common with *opd* genes from other sources, and has most likely undergone independent evolution (*132*).

The bacterial parathion-hydrolase genes (*opd*) have been cloned from *P. diminuta* (*133*, *134*) and *Flavobacterium* ATCC 27551(*135*). The nucleotide sequence for the *Flavobacterium* and *P. diminuta opd* genes are identical (*134, 135*). The *opd* genes were poorly expressed in *E. coli*, and the *Flavobacterium* hydrolase was a much larger protein when expressed in *E. coli* compared to the native *Flavobacterium* hydrolase. When the hydrolase was expressed in *Streptomyces lividins*, it was of similar size to that produced in *Flavobacterium,* but was synthesized in larger quantities and was secreted extracellularly (*136*). Production of an extracellular hydrolase is ideal for remediation processes because detoxification does not require bacterial uptake.

Nitrile Hydrolysis in Plants

Nitrile groups are essential moieties in the phytotoxicology of the herbicides, bromoxynil (3,5-dibromo-4-hydroxybenzonitrile), cyanazine {2-[[4-chloro-6-(ethylamino)-1,3,5-triazin-2-yl]amino]-2-methylpropanenitrile}, and dichlobenil (2,6-dichlorobenzonitrile), and the fungicide chlorothalonil (2,4,5,6-tetrachloro-1,3-benzenedicarbonitrile). Initial enzymatic hydrolysis of the nitrile group produces an amide. The amide is subsequently converted to the carboxylic acid, which may be decarboxylated. This metabolic pathway occurs for bromoxynil in wheat (*137*) and for cyanazine in wheat, potato (*Solanum tuberosum*) and maize (*Zea mays* L.) (*138, 139*).

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch004

Nitrile Hydrolysis in Microorganisms

In bacteria, the cyano group of bromoxynil can also be hydroxylated to the respective carboxylate by several species: *Fexibacterium* sp. (*140*) and *Klebsiella pneumoniae* (*141*). The *K. pneumoniae* utilizes bromoxynil as a nitrogen source, rather than a carbon source, with 3,5-dibromo-4-hydroxybenzoate accumulating as an end-product. Alternatively, an oxidative pathway, mediated by pentachlorophenol-hydroxylase (flavin monooxygenase) from *Flavobacterium* sp. ATCC 39723 (currently classified as a *Sphingomonas*), directly liberates cyanide, forming dibromohydroquinone (*142*). Formation of the hydroquinone derivative, rather than the hydroxybenzoate derivative, renders bromoxynil more prone to complete mineralization. *Klebsiella* bromoxynil-nitrilase genes (*bxn*) have been cloned, sequenced, and the protein purified (*143*). The *bxn* genes have been expressed in plants, resulting in bromoxynil-tolerant plants (*144*). Commercial application of this technology is currently being used in cotton and potatoes to produce herbicide-resistant crops.

Role of Phosphatases and Sulfatases in Pesticide Degradation

There is limited literature available on the role of phosphatases and sulfatases in pesticide metabolism. The insecticide endosulfan [1,2,3,4,7,7-hexachlorobicyclo [2.2.1]-2-heptene-5,6-bisoxymethylene sulfite] is metabolized *via* both oxidative and hydrolytic mechanisms *in vitro* by the white rot fungus, *Phenerochaete chrysosporium* (*145*). Under both nutrient-rich and nutrient-limiting conditions, endosulfan is metabolized to endosulfan diol. This indicates a different metabolic route, catalyzed by a sulfatase rather than a lignin peroxidase. Other studies have shown that endosulfan diol is also formed by hydrolytic cleavage of endosulfan by static cultures of the fungus *Trichoderma sp.* (*146*). These observations also suggest a role for sulfatase in fungal metabolism of endosulfan.

Genetic Engineering of Crops for Bialaphos/Glufosinate Resistance

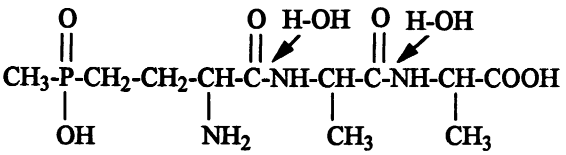
Numerous phytotoxic metabolites produced by microorganisms have been isolated, identified and tested for their potential as herbicides. Of these, bialaphos and phosphinothricin (PPT) are the most successful. Glufosinate (ammonium salt of phosphinothricin) and bialaphos have been developed as major commercial herbicides (see Figure 4). These compounds are included in this discussion because bialaphos is hydrolyzed by plant and microbial enzymes to yield the active herbicide (PPT), and also because PPT can be enzymatically acetylated at the primary amine moiety to yield the *N*-acetyl compound (non-phytotoxic) which may be acted on by hydrolases and/or transferases to yield the active phytotoxin (Figure 4). Furthermore, metabolism of the peptidyl compound bialaphos, and the resistance of transformed plants to phosphinothricin, are based on the presence or absence of transaminase and/or hydrolytic enzyme activity.

Bialaphos is a tripeptide comprised of a unique amino acid, L-2-amino-4-[hydroxy(methyl)phosphinyl]butyric acid (PPT) linked to two L-alanyl moieties. The compound was isolated from cultures of *Streptomyces viridochromogenes* (*147*), and *Streptomyces hygroscopicus* (*148*). The natural form of PPT is the L-isomer (L-PPT), and it was the first reported naturally-occurring amino acid containing a phosphinic group. Bialaphos was initially found to have some antifungal (*Botrytis cinerea*) and antibacterial activity (*147*, *149*), thought to be attributed to L-PPT. Glutamine reversed growth inhibition caused by bialaphos in *Bacillus subtilis* cultures (*147*). PPT also strongly inhibited glutamine synthetase [E.C. 6.3.1.2; GS] activity in *E. coli* (*147*). Later examination of L-PPT for phytotoxicity by Hoechst AG showed that it possessed strong phytotoxicity, and this compound was patented as a herbicide (*150*). Synthesis of the DL-PPT ammonium salt resulted in the commercial herbicidal formulation of this active ingredient. Bialaphos is rapidly degraded by microorganisms in soil to PPT (*151*), which is also rapidly degraded, with half-life of 4 to 7 days in soils (*152, 153*). In a test of 300 bacterial isolates from soil, all strains degraded L-PPT to the 2-oxo analog of PPT via transamination (*154*). Glufosinate (PPT) is a non-selective, postemergence herbicide used for weed control in orchards and vineyards, in chemical fallow situations, as a preharvest desiccant, as a burn-down herbicide of cover crops and/or weeds prior to no-till planting (*155*), and for weed control in transgenic crops resistant to the herbicide (*156*).

Bialaphos is absorbed through plant leaves, and some translocation (of bialaphos or its metabolites) occurs (*151*). Bialaphos is metabolized in plants soon after absorption to yield PPT. Bialaphos does not inhibit GS, but is rapidly metabolized by peptidases in plant tissues yielding PPT (*157*, *158*). The D-isomer of PPT is not a GS inhibitor (*159*), has no herbicidal activity (*156*), and is not acetylated in transgenic plants, which have been transformed with resistance to L-PPT (*160*). There is also a lack of degradative metabolism of L-PPT in non-transformed plants, but there is rapid conversion to the acetylated product in plants genetically altered for PPT resistance.

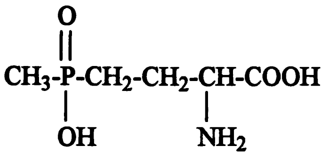
Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch004

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch004



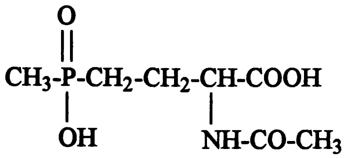
$$CH_3\text{-}\overset{\overset{O}{\|}}{\underset{\underset{OH}{|}}{P}}\text{-}CH_2\text{-}CH_2\text{-}\underset{\underset{NH_2}{|}}{CH}\text{-}\overset{\overset{O}{\|}}{C}\text{-}\overset{\overset{H\text{-}OH}{\swarrow}}{NH}\text{-}\underset{\underset{CH_3}{|}}{CH}\text{-}\overset{\overset{O}{\|}}{C}\text{-}\overset{\overset{H\text{-}OH}{\swarrow}}{NH}\text{-}\underset{\underset{CH_3}{|}}{CH}\text{-}COOH$$

Bialaphos



$$CH_3\text{-}\overset{\overset{O}{\|}}{\underset{\underset{OH}{|}}{P}}\text{-}CH_2\text{-}CH_2\text{-}\underset{\underset{NH_2}{|}}{CH}\text{-}COOH$$

Phosphinothricin



$$CH_3\text{-}\overset{\overset{O}{\|}}{\underset{\underset{OH}{|}}{P}}\text{-}CH_2\text{-}CH_2\text{-}\underset{\underset{NH\text{-}CO\text{-}CH_3}{|}}{CH}\text{-}COOH$$

***N*-Acetyl-phosphinothricin**

Figure 4. Chemical structures of bialaphos, phosphinothricin and N-acetylated phosphinothricin.

Biotechnological approaches have been utilized in studies on the biochemistry of PPT in microorganisms and plants. The biosynthetic pathway of bialaphos has been completely elucidated using various techniques (*161*). Beginning with precursors containing three carbon atoms, bialaphos is produced in a complex series of over a dozen steps (*161*). One step involves an acetyl CoA-dependent reaction that modifies either demethyl-PPT or PPT. The *bar* gene is responsible for resistance to bialaphos in *S. hygroscopicus*, and encodes for the acetyl transferase, which converts PPT to an acetylated non-phytotoxic metabolite (*162*). Although this acetyl transferase is not classified as a hydrolytic enzyme *per se*, it does form an amide bond that could therefore be susceptible to hydrolytic and/or transferase activity. As pointed out previously, deacetylases have been studied in plants, microorganisms, as well as in mammalian systems, and nitroacetanilide substrates have been utilized to facilitate assaying their activities (*163*). Such plant or microbial enzymes could act on *N*-acetyl-PPT to release the phytotoxic compound, PPT.

Over the past several years, many vegetable and cereal crop species have been transformed with genes imparting resistance to PPT. Cloning of a PPT-resistant gene (*bar*) from *S. hygroscopicus* (*164*) and the transformation of PPT-resistant plants has been accomplished (*165*). A similar gene (*pat*) from *S. viridochromogenes* Tü 494, with the same function, was simultaneously isolated and has also been introduced into various plant species (*160*, *166*, *167*). Presently, more than 20 crop plant species have been transformed for resistance to PPT in this manner. Some of these genetically altered plants are resistant to PPT at rates as high as 4 kg ha^{-1} (ca.10 times the lowest normal field application rate) (*164*). This indicates a high degree of incorporation of acetyl transferase expression.

The previously known anti-fungal activity of bialaphos and glufosinate was recently assessed on three pathogens (*Rhizoctonia solani, Sclerotinia homoeocarpa, and Pythium aphanidermatum*) *in vitro* and *in vivo* on PPT-resistant transgenic creeping bentgrass (*Agrostis palustris*), an important turfgrass (*168*). Results indicated that bialaphos can simultaneously control weeds and fungal pathogens in this transgenic grass. Furthermore, bialaphos has antibiotic activity against *R. solani* Kühn that causes rice sheath blight (*169*), and *Magnaporthe grisea* (Herbert) Barr (*148*) that causes rice blast disease. Substantial suppression of sheath blight symptoms was reported when bialaphos was applied to transgenic plants which had been infected with *R. solani* prior to herbicide treatment (*170*). Inoculated transgenic rice plants [bialaphos-resistant (*bar*) gene] had reduced lesions and other symptoms of rice blast disease after bialaphos treatment (*171*). It is assumed that these pathogens are controlled by bialaphos and PPT, because the microbes lack the ability to rapidly metabolize the compounds to non-fungitoxic products. Thus, it appears possible to control some serious diseases by using *bar*-transgenic rice cultivars and bialaphos for weed and disease control. Glufosinate has also been successfully used to control the weed red rice (a conspecific weed of cultivated rice) in *bar*-transformed rice (*172*).

Bialaphos and PPT are unique among commercial herbicides in that they have both potent antibiotic and herbicidal properties. This dual strategy will no doubt be utilized more widely with the increasing availability of PPT-resistant crops.

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch004

Summary and Conclusions

Generally, our understanding of microbial hydrolytic enzymes has been greatly increased during the past decade, but information on plant hydrolytic enzymes is not as advanced. Although advances have been made, most of the information on microbial hydrolytic enzymes, has not been focused directly on pesticide metabolism. Many hydrolytic enzymes have been reported to have multiple activities (amidase, esterase, transferase), but most have not been examined for multiplicity, especially with regard to the metabolism of pesticides. Also the knowledge about the precise physiological role of these hydrolytic enzymes is insufficient. Moreover, information is needed on enzyme mechanisms and regulation of enzyme activity. Many enzyme active sites or receptor sites recognize only one sterochemical geometry. Thus, understanding enzyme multiplicity, physiological role, mechanism, and regulation, may lead to the development of more specific regulators (e.g., inhibitors, activators), so that more specific and efficacious pesticidal compounds can be developed using a biorational design. The use of techniques such as protein engineering may provide additional insight on the relationship of protein structure and substrate specificities of hydrolytic enzymes in plants and microorganisms.

Many industrial synthetic processes produce racemic mixtures, in which only one enantiomer is biologically active. Hydrolytic enzymes have high potential value in the development of bioprocesses for production of compounds useful to agriculture and other industries. Enzymes have the unique ability to facilitate stereospecific transformations and thus, biosynthetic approaches may be more effective in some industrial syntheses. The cloning of an *Arthrobacter* esterase gene that stereospecifically produces (+) *t*-chrysanthemic acid, utilized in the synthesis of pyrethroid insecticides, is one example demonstrating this biotechnological strategy. This enzyme occurs in low amounts in this bacterium, however cloning and over-expression could permit industrial-scale preparation. Certain hydrolytic enzymes are also being considered for remediation of contaminants, e.g., nitrilases for solvents such as acetonitrile (*173*), amidases for acrylamides (*174*), and atrazine [6-chloro-*N*-ethyl-*N*-(1-methylethyl)-1,3,5-triazine-2,4-diamine] chlorohydrolase, to degrade atrazine (see chapter by Sadowsky and Wackett in this volume).

As we have discussed, crop engineering for resistance to herbicides, based upon microbial hydrolytic enzymes, is a commercial success for bialaphos and phosphinothricin. Future herbicide technologies may utilize other unique microbial hydrolytic enzymes that can be developed for engineering crop resistance to other herbicides. Other novel pesticides (fungicides, insecticides and herbicides) may also be designed as potent inhibitors of hydrolytic enzymes, or that would be activated or detoxified by specific plant or microbial hydrolytic enzymes.

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch004

References

1. Veerabhadrappa, P.S.; Montgomery, M. W. *Phytochemistry* **1971,** *10,* 1171-1174.

2. Incledon, B. J.; Hall, J. C. In *Regulation of Enzymatic Systems Detoxifying Xenobiotics in Plants*; Hatzios, K.K., Ed.; Kluwer Academic Press, The Netherlands, 1997; pp 67-82.
3. Bordenave, M.; Goldberg, R. Huet, J. C.; Pernollet, J. C. *Phytochemistry* **1995,** *38,* 315-319
4. Yaacoby, T.; Hall, J. C.; Stephenson, G. R. *Pestic. Biochem. Physiol.* **1991,** *41,* 296-304.
5. Jacobson, A.; Shimabukuro, R. H. *J. Agric. Food Chem.* **1984,** *32,* 742-746.
6. Brown, H. M.; Fuesler, T. P.; Ray, T. B.; Strachan, S. B. *Proc. 7th Intl. Congr. Pestic. Chem.*, VCH Verlagsgesellschaft, Weinheim, Germany **1991,** *1,* 257-266.
7. Brown, H. M.; Neighbors, S. M. *Pestic. Biochem. Physiol.* **1987,** *29,* 112-120.
8. Rusness, D. G.; Huwe, J. K.; Lamoureux, G. L. *Pestic. Biochem.PhysioL,* **1998,** *61,* 115-133.
9. Crafts, A.S. *J. Agric. Food Chem.* **1953,** *1,* 51-55.
10. Morré, D. J.; Rogers B. J. *Weeds* **1960,** *8,* 436-447.
11. Crafts, A. S. *Weeds* **1960,** *8,* 19-25.
12. Bates, A. N.; Spencer, D. M.; Wain, R. L. *Ann. Appl. Biol.* **1962,** *50,* 21-32.
13. Kloppenburg, D. J.; Hall, J. C. *Weed Res.* **1990,** *30,* 9-20.
14. Kloppenburg, D. J.; Hall, J. C. *Weed Res.* **1990,** *30,* 431-438.
15. Duke, S.O.; Kenyon, W. H. In *Herbicides: Chemistry, Degradation and Mode of Action;* Kearney, P. C.; Kaufman, D.D., Eds.; Marcel Dekker, Inc.; New York, NY, **1988,** pp 71-116.
16. Fedtke, C; Schmidt, R.R. *Weed Res.* **1977,** *17,* 233-239.
17. Hill, B. D.; Stobbe, E. H.; Jones, B. L. *Weed Res.* **1978,** *18,* 149-154.
18. Gorecka, K.; Shimabukuro, R. H.; Walsh, W. C. *Physiol. Plant.* **1981,** *53,* 55-63.
19. Tal, A.; Romano, M. L.; Stephenson, G. R.; Schwan, A. L.; Hall, J. C. *Pestic. Biochem. Physiol.* **1993,** *46,* 190-199.
20. Teresiak, H.; Street, J. E. *Proc. South. Weed Sci. Soc.* **1994,** *47,* 21-22.
21. Hoagland, R. E.; Zablotowicz, R. M.; Street, J. E. *2nd Internal. Weed Contr. Congr. Proc.*, Vol. III, **1996,** 819-826.
22. Frear, D.S. In *Herbicides: Chemistry, Degradation, and Mode of Action*; Kearney, P.C. and Kaufman, D.D., Eds.; Marcel Dekker, Inc.: New York, NY, 1975; Vol. 2, pp 541-607.
23. Köcher, H.; Kellner, H.M.; Lötzsch, K.; Dorn, E.; Wink, O. *Proc. Br. Crop Prot. Conf. Weeds.* **1982,** *1,* 341-347.
24. Smith, A.E.; Aubin, A.J. *Can. J. Soil Sci.* **1990,** *70,* 343-350.
25. Gaynor, J. D. *Soil Biol. Biochem.* **1992,** *35,* 29-32.
26. Wink, O.; Luley, U. *Pestic. Sci.* **1988,** *22,* 31-40.
27. Zablotowicz, R. M.; Hoagland, R.E.; Staddon, W. J.; Locke, M.A. *J. Agric. Food Chem.* **2000,** In press.
28. Gennari, M.; Vincenti, M.; Negre, M.; Ambrosoli, R. *Pestic. Sci.* **1995,** *44,* 299-303.

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch004

29. Hoagland, R. E.; Zablotowicz, R.M. *J. Agric. Food Chem.* **1998,** *45,* 4759-4765.
30. Choi, K. D.; Jeohn, G. H.; Rhee, J. S.; Yoo, O. J. *Agric. Biol. Chem.* **1990,** *54,* 2039-2045.
31. DeVries, R.P.; Michelsen, B.; Poulsen, C.H., Kroon, P.A.; van den Heuvel, R. H.; Faulds, C. B.; Williamson, G.; van den Hombergh, J. P.; Visser, J. *Appl. Environ. Microbiol.* **1997,** *63,* 4638-4644.
32. Politino, M.; Tonzi, S. M.; Burnett, W. V.; Romancik, G.; Usher, J. J. *Appl. Environ. Microbiol.* **1997,** *63,* 4807-4811.
33. Nishizawa, M.; Shimazu, M. Ohakawa, H.; Kanaoka, M. *Appl. Environ. Microbiol.* **1995,** *61,* 3208-3215.
34. McKay, D.B.; Jennings, M. P.; Godfrey, E. A.; MacRae, I. C.; Rogers, P. J.; Beachan, I. R. *J. Gen. Microbiol.* **1992,** *138,* 701-708.
35. Khalameyzer, V.; Fischer, I.; Bornscheuer, U. T.; Altenbuchner, J. *Appl. Environ. Microbiol.* **1999,** *65,* 477-482.
36. Brenner, S. *Nature* **1988,** *334,* 528-530.
37. Cygler, M.; Grochulski, P.; Schrag, J. B. *Can. J. Microbiol.* **1995,** *41* (Suppl 1), 289-296.
38. Feng, P. C. C.; Ruff, T. G.; Wangwala, S. H.; Rao, S. R. *Pestic. Sci.* **1998,** *59,* 89-103.
39. Neidert, K.; Van Epps, L.; Welch, W. *Pestic. Biochem. Physiol.* **1985,** *23,* 221-227.
40. Frear, D. S.; Still, G. G. *Phytochemistry* **1968,** *7,* 913-920.
41. Adachi, M.; Tonegawa, K.; Ueshima, T. *Noyaku Seisen Gijutsu* **1966,** *14,* 19-22.
42. Hoagland, R. E.; Graf, G. *Weed Sci.* **1972,** *20,* 303-305.
43. Hoagland, R. E.; Graf, G.; Handel, E. D. *Weed Res.* **1974,** *14,* 371-374.
44. Hoagland, R. E.; Graf, G. *Phytochemistry* **1972,** *11,* 521-527.
45. Hoagland, R. E. *Phytochemistry* **1975,** *14,* 383-386.
46. Hoagland, R. E. *Plant Cell Physiol.* **1978,** *19,* 1019-1027.
47. Smith, Jr., R. J.; Flinchum, W.T.; Searman, D. E. *U.S. Dept. of Agric., Agric. Handbook No. 497,* **1977,** 78pp.
48. Jun, D. J.; Matsunaka, S. *Pestic. Biochem. Physiol.* **1990,** *38,* 26-33.
49. Carey, V. F. III; Hoagland, R. E.; Talbert, R.E. *Weed Technol.* **1995,** *9,* 366-372.
50. Carey, V. F. III; Duke, S.O.; Hoagland, R. E.; Talbert, R.E. *Pestic. Biochem. Physiol.***1995,** *52,* 182-189.
51. Carey, V. F. III; Hoagland, R. E.; Talbert, R.E. *Pestic. Sci.* **1997,** *49,* 333-338.
52. Leah, J.N.; Caseley, J. C.; Riches, C. R.; Valverde, B. *Pestic. Sci.* **1994,** *42,* 281-289.
53. Oyamanda, M.; Tanaka, T.; Takasawa, Y.; Takematsu, T. *J. Pestic. Sci.* **1986,** *11,* 197-203.
54. Akatsuka, T. *Weed Res. (Japan)* **1979,** *24,* 55-63.
55. Walden, A.R.; Walter, C.; and Gardner, R. C. Gene Bank Protein Query Accesion # AAD04946.

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch004

56. Hoagland, R. E.; Zablotowicz, R. M.; Locke, M. A. In *Bioremediation Through Rhizosphere Technology*; Anderson, T.A., Coats, J.R. Eds.; ACS Symp. Ser. *563,* Amer. Chem. Soc. Washington DC, 1994, pp 160-179.
57. Wright, S. J. L.; Maule, A. *Pestic. Sci.* **1982,** *13,* 253-256.
58. Pelsey, F.; Leroux, P.; Helsot, H. *Pestic. Biochem. Physiol.* **1987,** *27,* 182-188.
59. Englehardt, G.; Wallnöfer, P. R.; Plapp, R. *Appl. Microbiol.* **1973,** *26,* 709-718.
60. Mochida, K.; Nakamura, T.; Li, W.X.; Ozoe, Y. *J. Pestic. Sci.* **1993,** *18,* 211-216.
61. Reichel, H.; Sisler, H. D.; Kaufman, D. D. *Pestic. Biochem. Physiol.* **1991,** *39,* 240-250.
62. Lanzillotta, R. P.; Pramer, D. *Appl. Microbiol.* **1970,** *19,* 307-313.
63. Wright, S. J. L.; Staintrope, A. F.; Downs, J. D. *Acta Phytopathol. Hung.* **1977,** *12,* 51-70.
64. Shabari, N. E. D.; Bordeleaux, L. M. *Appl. Microbiol.* **1969,** *18,* 369-375.
65. Hammond, M.; Price, C. P.; Scaven, M. D. *Eur. J. Biochem.* **1983,** *132,* 651-655.
66. Hoagland, R. E.; Zablotowicz, R. M. *Pestic. Biochem. Physiol.* **1995,** *52,* 190-200.
67. Hirase, K.; Matsunaka, S. *Pestic. Biochem. Physiol.* **1991,** *39,* 302-308.
68. Kearney, P. C.; Kaufman, D. D. *Science* **1965,** *147,* 740-741.
69. Blake, J.; Kaufman, D.D. *Pestic. Biochem. Physiol.* **1975,** *5,* 305-313.
70. Berger, B. M. *Pestic. Biochem. Physiol.* **1998,** *60,* 71-82.
71. Cullington, J. E.; Walker, A. *Soil Biol. Biochem.* **1999,** *31,* 677-686.
72. Bartha, R.; Pramer, B. *Science* **1967,** *156,* 1617-1618.
73. Bartha, R. *J. Agric. Food Chem.* **1968,** *16,* 602-604.
74. Zablotowicz, R.M.; Locke, M. A.; Hoagland, R. E.; Knight, S. S.; Cash, B. *Environ. Toxicol.,* **2000,** In press.
75. Yoshioka, H.; Nagasawa, T.; Yamada, H. *Eur. J. Biochem.* **1991,** *199,* 17-24.
76. Alt, J.; Heymann, E.; Krisch, K. *Eur. J. Biochem.* **1975,** *53,* 357-369.
77. Fournand, D.; Bigey, F.; Arnaud, A. *Appl. Environ. Microbiol.* **1998,** *64,* 2844-2852.
78. Zablotowicz, R.M.; Hoagland, R.E.; Wagner, S.C. *Soil. Biol. Biochem.* **1998,** *30,* 679-686.
79. Tiedje, J. M., Hagedorn, M. L. *J. Agric. Food Chem.* **1975,** *23,* 77-81.
80. McGahen, L. L.; Tiedje, J. M. *J. Agric. Food Chem.* **1978,** *26,* 414-416.
81. Villareal, D. T., Turco, R. F.; Konopka, A. *Appl. Environ. Microbiol.* **1991,** *57,* 2135-2140.
82. Martin, M.; Menes, G.; Allende, J. L.; Fernandez, J.; Alonso, R.; Ferrer, E. *Appl. Environ. Microbiol.* **1999,** *65,* 802-806.
83. Riley, P. S.; Behal, F. J. *J. Bacteriol.* **1971,** *108,* 809-816.
84. Shimizu, S.; Ogawa, J.; Chung, M. C. Yamada, H. *Eur. J. Biochem.* **1992,** *209,* 375-382.
85. Chebrou, H.; Bigey, F.; Arnaud, A.; Galzy, P. *Biochem. Biophys. Acta* **1996,** *1298,* 285-293.
86. Sorci, J.J.; Macalady, D.L. *J. Agric. Food Chem.* **1993,** *41,* 1760-1766.

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch004

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch004

87. Villarreal, D. T.; Turco, R. F.; Konopka, A. *Appl. Environ. Microbiol.* **1994,** *60,* 3939-3944.
88. Walker, A.; Brown, P.A. *Bull. Environ. Contam. Toxicol.* **1985,** *34,* 143-149.
89. Athiel, P.; Alfizar; Mercadier, C.; Vega, D.; Bastide, J.; Davet, P.; Brunel, B.; Cleyet-Marel, J.-C. *Appl. Environ. Microbiol.* **1995,** *61,* 3216-3220.
90. Mercadier, C.; Vega, D., Bastide, J. *FEMS Microbiol. Ecol.* **1997,** *23,* 207-215.
91. Walker, A. *Pestic. Sci.* **1987,** *21,* 219-231.
92. Matsunaka, S. *Science* **1968,** *160,* 1360-1361.
93. Kaufman, D. D.; Blake, J.; Miller, D. E. *J. Agric. Food Chem.* **1971,** *19,* 204-206.
94. Bowling, C. C.; Hudgins, H. R. *Weeds* **1966,** *14,* 94-95.
95. Norsworthy, J.K.; Talbert, R. E.; Hoagland, R. E. *Weed Sci.* **1999,** *47,* 13-19.
96. Hirase, K.; Matsunaka, S. *Proc. Br. Crop Prot. Conf. - Weeds* **1989,** *9,* 419-426.
97. Schlagbauer, A.W.J.; Schlagbauer, B.G.L. *Residue Rev.* **1972,** *42,* 1-84.
98. Schlagbauer, B.G. L.; Schlagbauer, A. W. J. *Residue Rev.* **1972,** *42,* 85-90.
99. Dyer, E.; Wright, G. C. *J. Amer. Chem. Soc.* **1959,** *81,* 2138-2143.
100. Still, G.G.; Mansager, E. R. *Phytochemistry* **1972,** *11,* 515-520.
101. Still, G.G.; Mansager, E. R. *Pestic. Biochem. Physiol.* **1973,** *3,* 87-95.
102. Lamoureux, G.L., Rusness, D.G. *J. Agric. Food Chem.* **1987,** *35,* 1-7.
103. Lay, M.-M.; Casida, J. E. *Pestic. Biochem. Physiol.* **1976,** *6,* 442-456.
104. Casida, J. E., Kimmel, E. C.; Ohkawa, H.; Ohkawa, R. *Pestic. Biochem. Physiol.* **1975,** *5,* 1-11.
105. Williams, I. H.; Pepsin, H.S.; Brown, M. J. Bull. *Environ. Contam. Toxicol.* **1976** *14,* 244-249.
106. Mateen, A.; Chapalamadugu, S.; Kaskar, B.; Bhatti, A.R.; Chaudhry, G. R. In *Biological Degradation and Bioremediation of Toxic Chemicals*, Chaudhry, G. R. Ed.; Dioscorides Press, Portland, OR, 1994, pp 198-233.
107. Parkin, T. B., Shelton, D. R.; Robinson, J. A. *J. Environ. Qual.* **1991,** *20,* 763-769.
108. Goldman, L. R.; Beller, M.; Jackson, R. J. *Arch. Environ. Health* **1990,** *45,* 141-147.
109. Talebi, K.; Walker, C. H. *Pestic. Sci.* **1994,** *42,* 37-42.
110. Kearney, P.C. *J. Agric. Food Chem.* **1965,** *13,* 561-564.
111. Pohlenz, H.-D.; Boidol, W.; Schuttke, I. *J. Bacteriol.* **1992,** *74,* 6600-6607.
112. Derbyshire, M. K.; Karns, J. S.; Kearney, P. C.; Nelson, J. O. *J. Agric. Food Chem.* **1987,** *35,* 871-877.
113. Mulbry, W.W.; Eaton, R.W. *Appl. Environ. Microbiol.* **1991,** *57,* 3679-3682.
114. Karns, J.S.; Tomasek, P. H. *J. Agric. Food Chem.* **1991,** *39,* 1004-1008.
115. Streber, W. R.; Kutschka, U.; Thomas, F. Pohlenz, H. D. *Plant Mol. Biol.* **1994,** *25,* 977-987.
116. Chapalamadugu, S.; Chaudhry, G. R. *Appl. Environ. Microbiol.* **1991,** *57,* 744-750.
117. Wilson, R.G. *Weed Sci.* **1984,** *32,* 264-268.
118. Lee, A. *Soil Biol. Biochem.* **1984,** *16,* 529-531.
119. Behki, R. M.; Khan, S.U. *Chemosphere* **1991,** *21,* 1457-1462.

120. McClung, G.; Dick, W. A.; Karns, J. S. *J. Agric. Food Chem.* **1994,** *42,* 2926-2931.
121. Dick, W. A.; Ankumah, R.A.; McClung, G, Abou-Assaf, N. In *Enhanced Biodegradation of Pesticides in the Environment,* Racke, K. D.; Coats, J. R. Eds.; ACS Symp. Ser. No. 426, Amer. Chem. Soc. Washington, DC, 1990, pp 98-112.
122. Nagy, I.; Compernolle, F.; Ghys, K.; Vanderlyden , J.; DeMot, R. *Appl. Environ. Microbiol.* **1995,** *61,* 2056-2060.
123. Shao, Z. Q.; Behki, R. M. *Appl. Environ. Microbiol.* **1995,** *61,* 2061-2065.
124. Shao, Z. Q.; Behki, R. M. *Appl. Environ. Microbiol.* **1996,** *62,* 403-307.
125. Rowlands, D. G. *J. Sci. Food Agric.* **1965,** *16,* 325-330.
126. Rowlands, D. G. *J. Sci. Food Agric.* **1966,** *17,* 90-93.
127. Bull, D. L.; Whitten, C. J.; Ivie, G. W. *J. Agric. Food Chem.* **1976,** *24,* 601-605.
128. Lamoureaux, G. L.; Frear, D.S. In *Xenobiotic Metabolism: In Vitro Methods,* Paulson, G. D.; Frear, D. S.; Marks, E. P., Eds.; ACS Symp. Ser. No. 97, Amer. Chem. Soc., Washington, DC., 1979, pp 77-128.
129. Mulbry, W.W.; Karns, J.S. *Appl. Environ. Microbiol.* **1989,** *55,* 289-293.
130. Lewis, V. E.; Donarski, W. J.; Wild, J. R.; Raushel, F.M. *Biochemistry* **1988,** *27,* 1591-1597.
131. Shelton, D. R.; Somich, C. J. *Appl. Environ. Microbiol.* **1988,** *54,* 2566-2571.
132. Mulbry, W. W. *Gene,* **1992,** *12,* 149-153.
133. McDaniel, C.S.; Harper, L.L.; Wild, J.R. *J. Bacteriol.* **1988,** *170,* 2306-2311.
134. Chaudhry, G.R.; Ali, A. N.; Wheeler, W. B. *Appl. Environ. Microbiol.* **1988,** *54,* 288-293.
135. Mulbry, W.W.; Karns, J.S. *J. Bacteriol.* **1989,** *171,* 6740-6746.
136. Steiert, J. G.; Pogell, B. M.; Speedie, M. K.; Laredo, J. *Bio/Technology* **1989,** *7,* 65-68.
137. Buckland, J.L.; Collins, R.F.; Pullin, E.M. *Pestic. Sci.* **1973,** *4,* 149-162.
138. Benyon, K.I.; Stoydin, G.; Wright, A.N. *Pestic. Sci.* **1972,** *3,* 293-305.
139. Benyon, K.I.; Stoydin, G.; Wright, A.N. *Pestic. Sci.* **1972,** *3,* 379-387.
140. Cullimore, D. R.; Kohoup, M. *Can. J. Microbiol.* **1974,** *20,* 1449-1452.
141. McBride, K.E.; Kenny, J. W.; Stalker, D. N. *Appl. Environ. Microbiol.* **1986,** *52,* 325-330.
142. Topp, E.; Xun, L.; Orser, C. S. *Appl. Environ. Microbiol.* **1992,** *58,* 502-507.135.
143. Stalker, D.M.; Malyj, L. B.; McBride, K. E.; *J. Biol. Chem.* **1988,** *263,* 6310-6314.
144. Stalker, D.M.; McBride, K. E.; Malyj, L. B. *Science* **1988,** *242,* 419-423.
145. Kullman, S.W.; Matsumura, F. *Appl. Environ. Microbiol.* **1996,** *62,* 593-600.
146. Katayama, A.; Matsumura, F. *Environ. Toxicol. Chem.* **1993,** *12,* 1059-1065.
147. Bayer, E.; Gugel, K. H.; Hägele, K.; Hagenmaier, H ; Jessipow, S.; König, W. A.; Zähner, H. *Helv. Chim. Acta* **1972,** *55,* 224-239.

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch004

148. Kondo, Y.; Shomura, T.; Ogawa, Y.; Tsuruoka, T.; Watanabe, H.; Totsukawa, K.; Suzuki, T.; Moriya, C.; Yoshida, J. *Sci. Rept. Meiji Seika Kaisha* **1973**, *13*, 34-44.
149. Niida, T.; Inouye, S.; Tsuruoka, T.; Shomura, T.; Kondo, Y.; Ogawa, Y.; Watanabe, H.; Sekizawa, Y.; Watanabe, T.; Igarashi, H. Germ. Offen. DE 2 236 599, 1973.
150. Rupp, W.; Finke, M.; Bieringer, H.; Langelueddeke, P. Germ. Offen. DE 2 717 440, 1977.
151. Tachibana, K.; Watanabe, T.; Sekizawa, Y.; Takematsu, T. *J. Pestic. Sci.* **1986**, *11*, 27-31.
152. Gallina, M. A.; Stephenson, G. R. *J. Agric. Food Chem.* **1992**, *40*,165-168.
153. Smith, A.E. *J. Agric. Food Chem.* **1988**, *36*, 393-397.
154. Bartsch, K.; Tebbe, C. *Appl. Environ. Microbiol.* **1989,** *55,* 711-716.
155. American Hoechst Corp. 1982, HOE-00661 Technical Information Bulletin. AMF 2464. Somerville, NJ.
156. Vasil, I.K. In *Herbicide-Resistant Crops;* Duke, S.O., Ed.; CRC Press: Boca Raton, FL, 1996; pp 85-91.
157. Tachibana, K. *Pestic. Sci. Biotechnol. Proc., 6th (1986) Internatl. Congr. Pestic. Chem.,* **1987,**145-148.
158. Wild, A.; Ziegler, C. *Z. Naturforsch.* **1989**, *44c,* 97-102.
159. Manderscheid, R.; Wild, A. *J. Plant Physiol.* **1986**, *123*, 135-142.
160. Dröge, W.; Broer, I ; Pühler, A. *Planta* **1992**, *187*, 142-151.
161. Murakami, T.; Anzai, H.; Imai, S.; Satoh, A.; Nagaska, K.; Thompson, C. *J. Mol. Gen. Genet.* **1986,** *205*, 42-50.
162. Kumada, Y.; Anzai, H.; Takano, E.; Murakami, T.; Hara, O.; Itoh, R.; Imai, S.; Satoh, A.; Nagoaka, K. *J. Antibiot.* **1988,** *41*,1839-1845.
163. Hoagland, R. E.; Graf, G. *Enzymologia* **1971,** *41,* 313-319.
164. Thompson, C. J.; Movva, N. R.; Tigard, R.; Crameri, R.; Davies, J. E.; Lauwereys, S. M.; Botterman, J. *EMBO J.* **1987,** *6*, 2519-2523.
165. DeBlock, M.; Botterman, J.; Vanderwiele, M ; Dockz, J.; Thoen, C.; Gossele, V.; Movva, N. R.; Thompson, C.; van Montagu, J.; Leemans, J. *EMBO J.* **1987,** *6*, 2513-2518.
166. Broer, I ; Arnold, W.; Wohlleben, W.; Pühler, A. In *Proc. Braunschweig Symp. Applied Plant Molecular Biol.*, Galling: Braunschweig; Germany, 1989; p 240.
167. Wohlleben, W.; Arnold, W.; Broer, I.; Hillemann, D.; Strauch, E.; Pühler, A. *Gene* **1988**, *70*, 25-37.
168. Liu, C. A.; Zhong, H.; Vargas, J.; Penner, D.; Sticklen, M. *Weed Sci.* **1998**, *46*, 139-146.
169. DeDatta, S. K. *Principles and Practices of Rice Production;* Wiley International: New York, NY, 1981.
170. Uchimiya, H.; Iwata, M.; Nojiri, C.; Samarajeewa, P.K.; Takamatsu, S.; Ooba, S.; Anzai, H.; Christensen, A.H.; Quail, P.H.; Toki, S. *Bio/Technology* **1993,** *11*, 835-836.
171. Tada, T.; Kanzaki, H.; Norita, E.; Uchimiya, H.; Nakamura, I. *Molec. Plant-Microbe Interact.* **1996**, *9*, 762-764.

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch004

172. Sankula, S.; Braverman, M. P.; Jodari, F.; Linscombe, S. D.; Oard, J. H. *Weed Technol.* **1997**, *11*, 70-75.
173. Babu, G. R.; Wolfram, J. H.; Marian, J. M.; Chapatwala, K. D. *Appl. Microbiol. Biotechnol.* **1995,** *43,* 739-745.
174. Nawaz, M. S.; Khan, A. A.; Seng, J. E.; Leakey, J. E.; Siitonen, P. H.; Cerniglia, C. E. *Appl. Environ. Microbiol.* **1994,** *60,* 3343-3348.

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch004

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch005

Chapter 5

Biochemical Conjugation of Pesticides in Plants and Microorganisms: An Overview of Similarities and Divergences

J. Christopher Hall, J. Sue Wickenden, Kerrm Y. F. Yau

Department of Environmental Biology, University of Guelph, Guelph, Ontario N1G 2W1, Canada

Metabolic systems protect organisms from toxic substances. Conjugation reactions, found in fungi, bacteria and plants, not only detoxify metabolic wastes, but also form structural molecules and act to regulate hormone action. Conjugation has been defined by Dorough (1976) as a metabolic process whereby endogenous and exogenous chemicals are converted to polar components facilitating their removal from site(s) of continuing metabolism. However, pesticide conjugates are not always made more polar e.g., 2,4-D-leucine in soybean (*Glycine max*) and methylated arsenic and mercuric compounds in microbes are less polar. Generally, enzymes involved in conjugation are not substrate-specific, i.e., they detoxify both exogenous and endogenous compounds. There are many differences among the types of conjugates found in plants and soil microbes (fungi, bacteria). In plants, sugar and amino acid conjugates are formed, whereas, in nutrient-limited soil microbes, sugars and amino acids are rarely available for conjugation. Microbes use different endogenous substrates produced from continuing metabolism, e.g. methyl and acyl conjugates from methanogenesis. Conjugation reactions also confer herbicide selectivity in plants. These conjugates may be moved into the vacuole and/or incorporated in the cell wall matrix/vascular tissue. Fate of pesticides in soil may also be affected by conjugation. Conjugates may be immobilized becoming biologically unavailable, or made more recalcitrant and lipophilic and accumulate in the food chain.

In Pesticide Biotransformation in Plants and Microorganisms; Hall, J., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2000.

Introduction

Currently, there are over 900 pesticide products, including herbicides, insecticides and fungicides, with over 600 active ingredients. Despite the improvements in selectivity and precision application techniques, it is estimated that as little as 0.1 to 5% of a herbicide may reach its target weed and 0.003% of an insecticide may be consumed by the target pest (*1*). Considering the smaller size of fungal targets, the amount of fungicide reaching them will be proportionally even smaller (*2*).

Conjugation is one of the most important detoxification mechanisms found in living organisms. Dorough (*3*) defined conjugation as a metabolic process whereby endogenous and exogenous chemicals are converted to polar components to facilitate their removal from the site(s) of continuing metabolic processes. Conjugation reactions found in most plants fulfill this definition. Exogenous xenobiotics are detoxified by the addition of a natural substance that is present inside the plant cells (i.e. endogenous). The reaction changes the chemical properties of the xenobiotic and renders it unavailable to the primary metabolism of the plant. Addition of either a sugar, an amino acid or a glutathione moiety to the xenobiotic results in a conjugate with a higher molecular weight that is more water-soluble, less mobile, and is usually more susceptible to further processing in the plant, all of which makes it unreactive towards the target site. However, there are also conjugates that are less polar than the parent compound that remain phytotoxic.

Some microbially derived conjugates of pesticides, however, have different chemical properties than their counterparts in plants. Conjugation reactions occur in fungi and bacteria as one of their many detoxification mechanisms. In this chapter, we compare the different conjugation reactions of pesticides in plants, fungi, and bacteria, and attempt to extend the definition of conjugation to cover this process in all these organisms. We propose that conjugation of a pesticide should be extended to include those metabolic processes in which a product from natural metabolism is incorporated into a pesticide or its metabolite to facilitate the compartmentalization, sequestration, detoxification and/or mineralization of the compound. Furthermore, we propose that conjugation also occurs outside the microorganism when enzymes and/or substrates from the organism are available.

There are several similarities between conjugation reactions in plants and microorganisms. In both groups of organisms, conjugation is a metabolic process that uses existing enzymatic machinery to detoxify xenobiotics; new enzymes are not synthesized. This type of metabolism is called co-metabolism. Natural substrates are converted to provide energy and/or a carbon source for primary metabolism, and simultaneously the xenobiotics are transformed into other compounds (*4*). The latter transformations do not provide nutrients or energy to the living organisms, however, the organisms conserve energy and resources by not building new pathways for the detoxification of different xenobiotics in which they are exposed. Moreover, co-

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch005

metabolism will provide plants and microbes with a survival advantage over those populations lacking the constitutive enzymes for pesticide detoxification.

Conjugation reactions found in plants and microorganisms are shown in Table 1. Some of the reactions, such as glycosylation and amino acid conjugations, are dominant in plants where glucose and amino acids are abundant. On the other hand, fungi and bacteria are heterotrophs and compete for nutrients in the soil environment, where glucose and amino acids are metabolized as soon as they are available, so these types of reactions only occur under controlled laboratory conditions. Conjugation of pesticides to glucose or amino acids for the purpose of detoxification would be metabolically expensive for soil microbes.

Table I. Xenobiotic conjugation reactions recognized or presumed to occur in plants and microorganisms (Adapted from ref 5.)

Type of Conjugates	Plants	Microorganisms
Glucosides/Glycosides	++	+
Amino Acid	++	?
Glutathione	++	+
Acetyl, Formyl	++	+
Methyl	+	+

Legend:		
	++	Conjugate formation demonstrated with several xenobiotics
	+	Conjugation reaction found with particular xenobiotics
	?	Conjugate formation presumed to occur from the presence of enzymes and/or natural conjugates

Conjugates of plants and microorganisms have different fates. Plants lack an excretory system, so their toxic products are usually compartmentalized in vacuoles or embedded in structural polymers to prevent pesticide toxicity. However, in some cases, small quantities of pesticide conjugates can be exuded from the roots. Generally, the conjugates formed will be stored permanently and never be further metabolized. Some microbes have a different strategy, they prevent toxic compounds from entering the cells, for example, extracellular enzymes of fungi conjugate xylose to phenols. Some conjugates that are formed inside microorganisms are less toxic than their parent compounds so they are not as harmful when they are stored in the cytoplasm. Some conjugates are further mineralized to their inorganic components by existing metabolic pathways and thus detoxified.

In the following sections, different conjugation reactions in plants and microorganisms are described. Some reactions are presented to highlight the variations in conjugation reactions, thereby compelling reconsideration of Dorough's 1976 definition.

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch005

Types of Pesticide Conjugations in Plants

Detoxification of pesticides by conjugation in plants often involves addition of either a sugar or a glutathione moiety to the herbicide molecule. The resulting conjugate has a higher molecular weight, is more water-soluble, less mobile, and is usually more susceptible to further processing in the plant. These factors render the compound unreactive at the target site. Xenobiotics can also be conjugated to amino acids, a process that may yield more toxic conjugates (*6*).

There are generally three phases of herbicide metabolism in plants. Prior to conjugation, most herbicides undergo a phase I metabolic reaction, which is oxidative, reductive or hydrolytic. Phase I reactions result in the formation of free amino, hydroxyl or carboxylic acid groups. An enzymatic addition of a sugar, amino acid or glutathione molecule may occur at the newly formed functional group produced during phase I; this type of reaction occurs in Phase II metabolism. Finally, phase III metabolism either converts phase II conjugates to insoluble residues or conjugates them to an additional molecule. It is likely that plants evolved phase III metabolism to further detoxify xenobiotics because phase II conjugates cannot be excreted from plants in significant levels (*7, 8*). These phases of plant metabolism usually result in the detoxification of xenobiotics or prevent them from interacting with their target sites in the plant. Recently, a revision to the phase classification of herbicide metabolism has been proposed (*9, 10*). This classification consists of four steps, which are called functionalization, conjugation, processing and compartmentalization.

Several types of conjugation reactions occur in plants, as described below. Extensive research has been conducted in the area of glutathione conjugation, however, less is known about glucose and amino acid conjugation.

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch005

Glucose Conjugation

In plants, glucose can be added to xenobiotics via several types of reactions. The six types of glucose conjugates that will be discussed include *O*-, *N*-, and *S*-glucosides, glucose esters, gentiobiosides and malonyl conjugates.

Herbicides with hydroxylated aromatic rings can be conjugated with sugars to form *O*-glucosides. The β-*O*-D-glucoside of bentazon formed in soybean and rice (*Oryza sativa*) is an example of an *O*-glucoside (Figure 1), where glucose is added to the hydroxyl group of 6-OH bentazon, which was derived from phase I metabolism. *O*-glucosides are the most common glucosides formed, probably since many oxidation reactions involve the addition of oxygen. Similar conjugation reactions also occur at amino groups on the aromatic ring of xenobiotics to form *N*-glucosides, i.e. the *N*-glucoside of chloramben (*11*). Conjugation reactions occurring at sulfhydryl groups yield the less common S-glucosides, e.g. an S-glucoside of the fungicide dimethyldithiocarbamate (*12*).

Although plants are capable of forming galactose, glucuronic acid and other carbohydrate conjugates with endogenous substrates such as flavones (*13*), monosaccharide conjugates of xenobiotics involving carbohydrate moieties other than glucose are rare (*14*). The frequency of glucose conjugation may be due to

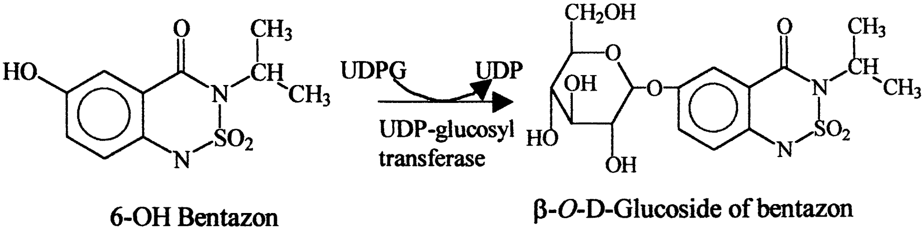


Figure 1. Formation of the O-glucoside of bentazon in rice (Adapted from ref 15).

the relatively high concentration of pre-existing uridine diphosphate glucose (UDPG) in plant cells (*16*). UDPG functions primarily as the immediate glucose donor in cellulose biosynthesis (*17*). The majority of glucosyl transferase enzymes involved in conjugation reactions are specific for UDPG.

UDPG is also used as a donor for the formation of glucose esters, but in this case glucose is added to a carboxylic acid group of a xenobiotic (Figure 2). Esterases in the cytoplasm can reverse this reaction. For example, glucose esters of chloramben are reactive and readily hydrolyzed back to the parent herbicide (*18*). Some glucose esters can undergo a second conjugation with another glucose molecule to yield a gentiobioside (*19*). However, the second sugar that is transferred to the glucose conjugate does not have to be glucose. For example, a disaccharide conjugate of 3-phenoxybenzyl alcohol was isolated from cotton (*Gossypium* spp.) leaves where the first attached sugar was glucose and the second was arabinose (*20*). Conjugates containing sugars other than glucose, such as arabinose, are called glycosides, whereas glucose conjugates are called glucosides (*21*).

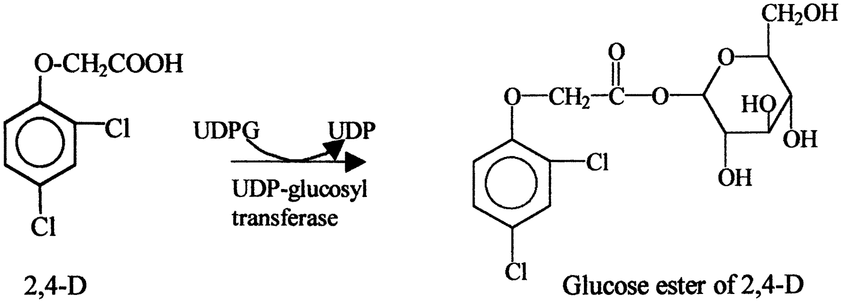


Figure 2. Formation of the glucose ester of 2,4-D in wheat (Adapted from ref 22).

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch005

Malonic Acid Conjugation

In phase III metabolism, glucose conjugates of xenobiotics are conjugated a second time to become malonic acid hemi-esters in the presence of malonyl CoA (*23*). This type of conjugation is thought to be a common route for conjugates of glycosylated xenobiotics in higher plants (*14*). Nine pesticides have been isolated from seven plants species as malonyl glucose conjugates.

In some cases, xenobiotics are conjugated with glutathione, which is catabolized to cysteine prior to its conjugation with malonate. For example, fluorodifen-GSH is metabolized to a cysteine conjugate and is subsequently *N*-acylated with malonic acid (*24*).

Glutathione Conjugation

Glutathione (GSH) is a tripeptide (γ-glutamylcysteinyl-β-glycine) found in aerobic organisms, and is involved in the conjugation of xenobiotics. The tripeptide homoglutathione, in which alanine is substituted for glycine, performs a similar function. Glutathione and homoglutathione conjugations occur with electrophilic xenobiotics to form less toxic and more polar molecules (*25, 26, 27*). Conjugation of xenobiotics with glutathione can occur enzymatically via glutathione-S-transferases (GST) or non-enzymatically, although enzymatic conjugations occur at a faster rate (*28*). For example, GST causes ether bond cleavage in fluorodifen, yielding a GSH conjugate and *p*-nitrophenol (Figure 3). Plants with high concentrations of GSTs are more tolerant to certain herbicides than plants with a lower enzyme titer (*29, 30*). For example, cotton, peanut (*Arachis hypogaea*) and soybean, which are resistant to fluorodifen, have higher GST activity than susceptible tomato (*Lycopersicon esculentum*) and cucumber (*Cucumis sativus*) species (*31*).

Grass species that are moderately tolerant (i.e. wheat, *Triticum aestivum*) or moderately resistant (i.e. barley, *Hordeum vulgare*) to fenoxaprop-ethyl have higher levels of GSH and cysteine than susceptible species such as oat (*Avena sativa*) or yellow foxtail (*Setaria glauca*) (*30*). In all species studied, GST activity was very low. It was hypothesized that fenoxaprop-ethyl is activated by enzymatic de-esterification to fenoxaprop. In wheat and barley, fenoxaprop is cleaved at the ether bond non-enzymatically and conjugated to GSH. In susceptible species, low levels of GSH limit fenoxaprop-GSH conjugation and thus prevent detoxification of the herbicide.

Following GSH-conjugation of xenobiotics in the cytoplasm, the resulting conjugates must be removed to maintain GST activity levels. An ATP-dependent pump that recognizes the glutathione moiety of conjugates is responsible for conjugate transport from the cytoplasm into the vacuole (*32*). The ATP pump is very similar to a mammalian plasma membrane GSH-conjugate transporter, since it requires magnesium and is inhibited by vanadate. The plant vacuolar pump transports many glutathione conjugates, for example *N*-ethylmaleamide-GSH,

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch005

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch005

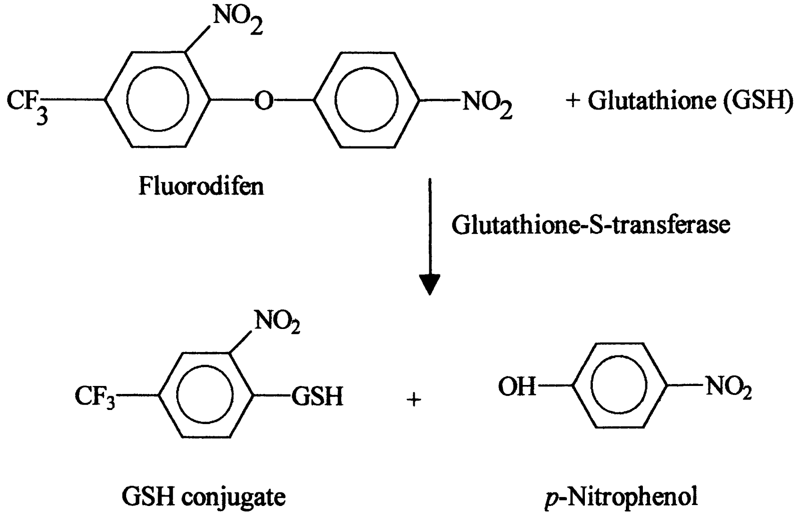


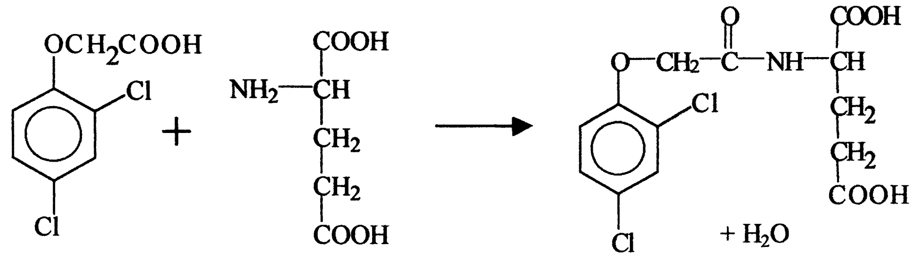
Figure 3. Enzymatic conjugation of flurodifen to glutathione in pea. (Adapted from ref 24).

metolachlor-GSH, simetryn-GSH and dinitrobenzene-GSH. No degradation of conjugates occurred over the duration of study (*32*). The biosynthesis of glutathione, its subsequent conjugation to a xenobiotic, and transfer of the conjugate into the vacuole must all be efficient enough to provide adequate protection against herbicide influx into the cell. The effectiveness of this GST/GSH detoxification mechanism may be the basis for the selectivity and resistance of many plant species to herbicides (*33*).

Amino Acid Conjugation

Types of Metabolites

Feung et al. (*34*) examined the metabolites of 2,4-D from soybean callus. After 48 hrs of incubation with [1-^{14}C]-2,4-D, soybean callus tissue absorbed nearly all of the radiolabelled herbicide. Of the absorbed herbicide, 17-23% was found in the water-soluble fraction of the callus tissue, 70-76% in the ether-soluble fraction and 3-6% as insoluble residues. In the water-soluble fraction, 4-OH-2,5-D, 5-OH-2,4-D and traces of parent 2,4-D were present. This parent 2,4-D was theorized to have been produced following hydrolysis of the glucose ester of 2,4-D. In the ether-soluble fraction, the metabolites were 2,4-D and 2,4-D-glutamic acid (2,4-D-glu) (Figure 4). Characterization of the insoluble residue was not possible (*32*).



2,4-D Glutamate 2,4-D-glu

Figure 4. Amino acid conjugation in pea (Pisum sativum). (Adapted from ref 35).

In later research, six additional amino acid conjugates of 2,4-D were identified in soybean callus: 2,4-D-aspartate, 2,4-D-alanine, 2,4-D-valine, 2,4-D-leucine, 2,4-D-phenylalanine and 2,4-D-tryptophan. These amino acid conjugates of 2,4-D are phytotoxic, although less so than 2,4-D. Specific enzymes may exist to form 2,4-D-conjugates with a certain amino acid, or a more general set of enzymes with less specificity may exist. The amino acid or the substrate may have to be activated (step 1) before conjugation can occur (step 2). It has been theorized that one enzyme could catalyze both reactions (*36*).

1. Activation: 2,4-D + ATP $\Leftrightarrow$ 2,4-D-AMP + PPi
2. Conjugation: 2,4-D-AMP + amino acid $\Leftrightarrow$ 2,4-D-amino acid + AMP

Tobacco (*Nicotiana rustica*) cells rapidly conjugate glutamate to 2,4-D (*37*). 2,4-D hydroxylation occurs more quickly when 2,4-D-glu is the substrate rather than 2,4-D (*36*). If the hydroxylation is specific for certain amino acid conjugates, the plant cell may activate 2,4-D using ATP, followed by conjugation with glutamic acid (less toxic than 2,4-D) to ensure fast and complete detoxification to hydroxylated 2,4-D-glutamate.

The proposed activation/conjugation scheme closely parallels the amino acid conjugation of xenobiotics in rats. In rats, acyl CoA synthetase 'activates' benzoic acid to benzoyl CoA in the presence of ATP and CoA. Then, glycine is conjugated to the activated benzoyl-CoA by Acyl-CoA:amino acid N-acyltransferase to produce the final product, hippuric acid (*38*).

Callus Versus Whole Plant

Much of the work on amino acid conjugation has been performed on callus tissue rather than on intact plants. Experimentally, callus tissue is practical because metabolite identification is faster and easier. Pigments and starches from whole

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch005

plants that can interfere with herbicide metabolite identification, are absent in heterotrophic cultures of plant callus tissue (*39*). 2,4-D metabolism in callus tissue and plants were compared. There were no qualitative differences between the ether-soluble or water-soluble fractions in either whole plants or callus (*40*). Differences were noted in the quantities of some of the metabolites. For example, amino acid conjugates in soybean callus were much more abundant than in soybean or corn plants, possibly reflecting an increased prevalence of inducible enzyme systems that form amino acid conjugates in callus cells (*40*). Both callus and whole plants were able to metabolize 2,4-D by ring hydroxylation and conjugation of the carboxylic acid group with either amino acids or sugars. Although relative percentages of metabolites vary in whole plants and callus, it was concluded that herbicides may be metabolized in a similar fashion (*40*).

Herbicidal Activity of Amino Acid Conjugates

All twenty amino acids were identified as conjugates of 2,4-D, all were biologically active, and stimulated both plant cell division and elongation (*6, 41*). All of the conjugates demonstrated herbicidal properties, but no specific conjugate exceeded the activity of 2,4-D on all plants tested (*42*). Non-polar amino acid conjugates such as 2,4-D-leucine and 2,4-D-methionine exhibited sufficient herbicidal activity to reduce crop yields. The non-polar properties of these amino acid conjugates may have enhanced cuticle permeability of the herbicide-conjugate (*42*). Conversely, amino acid conjugates such as 2,4-D-aspartate and 2,4-D-glutamate were shown to have poor herbicidal activity, due to increased water solubility, which impeded cuticle penetration. Therefore, 2,4-D-glutamate conjugation was interpreted to be a protective mechanism against 2,4-D damage, since it is one of the more common amino acid conjugates formed in plants (*36*).

Metabolism of 2,4-D Amino Acid Conjugates

Amino acid conjugates may be hydrolyzed to free 2,4-D and other water-soluble metabolites. When unlabelled 2,4-D was supplied to callus cells, it did not affect the rate of disappearance of 2,4-D amino acid conjugates but it did reduce the percentage of free [1-^{14}C]-2,4-D. Therefore, an external supply of 2,4-D does not directly prevent metabolism of amino acid conjugates of 2,4-D in soybean tissue culture. This implies that amino acid conjugates in callus cells are actively metabolized to free 2,4-D (*43*).

Prevention of Morphogenesis

Morphogenesis is the process of cellular differentiation into tissues and organs. Carrot (*Daucus carota*) cells are able to undergo morphogenesis after 3 days of incubation with [2-^{14}C]-2,4-D, whereas soybean callus cells do not undergo

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch005

morphogenesis (*44*). Carrot and soybean callus cells retained 30 and 70% of the radioactivity, respectively. The long time-course may account for the conjugation of 2,4-D to amino acids and sugars in soybean cells, thus facilitating compartmentalization of conjugates into the vacuole. Retention of 2,4-D and 2,4-D-conjugates by soybean callus cells may be responsible for the prevention of their morphogenesis.

Immobilization of Pesticides

Vacuolar Compartmentalization

The vacuole is enclosed by a Golgi-produced tonoplast membrane. Vacuoles are key organelles involved in both the storage of compounds and in osmoregulation. Substrate specific transport systems in the tonoplast membrane allow for the bi-directional passage of both organic compounds and inorganic ions. ATP-dependent pumps that transport glutathione conjugates into the vacuole play an important role in the sequestration of conjugates. No transporters of glucose or amino acid conjugates have been characterized, although they have been hypothesized to exist.

Herbicides are conjugated to endogenous plant metabolites in the cytoplasm of the cell where enzymes, such as UDP-glucosyl transferase and substrates, such as glucose and amino acids are located. A proposed natural metabolite of a conjugate that would aid transport through the lipophilic vacuolar membrane may be a malonyl group, which is common to many herbicide conjugates (*14*).

Several herbicide conjugates, such as glucose esters, are thought to be compartmentalized in the vacuole (pH 5.0–6.0) because they are more prone to hydrolysis by esterases in the cytoplasm (pH 7.0–7.5). When intact vacuoles from *Hippeastrum* flower petals were isolated and investigated, hydrolytic enzymes such as esterases or β-glucosidases were not found (*45*). The water-soluble portion of the vacuole did contain β-D-glucoside conjugates, which would be more stable in the vacuole due to the reported absence of the β-glucosidase enzyme (*46*).

Insoluble Residues

Detoxification of herbicides by binding to cell wall components is an example of phase III metabolism. Diuron was hydrolyzed to yield 3,4-dichloroaniline in the isolated lignin fraction of rice roots. Forty percent of the recovered [1-^{14}C]-3,4-dichloroaniline was thought to be bound covalently to lignin (*47*). The incorporation of chloroaniline herbicide residues into lignin effectively detoxifies the diuron by cell wall sequestration. An insoluble 2,4-D metabolite fraction was covalently bound to lignin and deposited in the cell wall (*48*).

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch005

Conjugation Imparts Selectivity

Chloramben Metabolism

Pesticides with two or more groups that are susceptible to phase I reactions, such as NH_2, -OH or –COOH groups, can form a range of conjugates. Chloramben metabolism can produce either a glucose ester or an *N*-glucoside (Figure 5).

Since glucose esters can be unstable, the toxic parent herbicide may be regenerated (*7*). To determine if this occurred with chloramben, excised soybean (tolerant) and barley (susceptible) tissues and seedlings were treated with either radioactive glucose ester conjugates or *N*-glucoside conjugates of chloramben (*18*). The *N*-glucoside remained unchanged in both species, but the glucose ester was hydrolyzed to yield chloramben, which was subsequently conjugated to an *N*-glucoside. In susceptible barley, the rate of *N*-glucoside biosynthesis was slower than in tolerant soybean, glucose ester biosynthesis appeared to compete for available chloramben (*18*). The reduced rate of *N*-glucoside biosynthesis lengthened the residence time of phytotoxic chloramben levels at target sites in the susceptible barley. Biochemical differences in the rate of *N*-glucoside biosynthesis as well as glucose ester formation and its hydrolysis appear to determine chloramben phytotoxicity in different species (*18*).

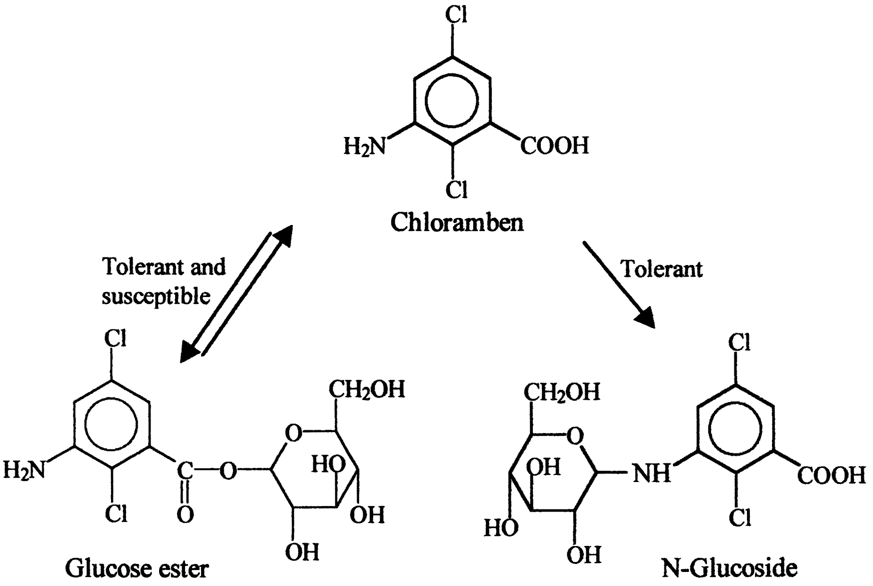


Figure 5. Metabolism of chloramben in a tolerant (e.g. soybean) and susceptible (e.g. barley) species (Adapted from ref 18).

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch005

Picloram Metabolism

Like chloramben, picloram has two substituent groups which can undergo phase I metabolism prior to conjugation. A metabolic pathway for picloram in leafy spurge (*Euphorbia esula*) was proposed (Figure 6). In this pathway, both the *N*-glucoside and glucose ester of picloram undergo further conjugation, i.e. the *N*-glycoside is conjugated with malonate and the glucose ester is converted to a gentiobioside.

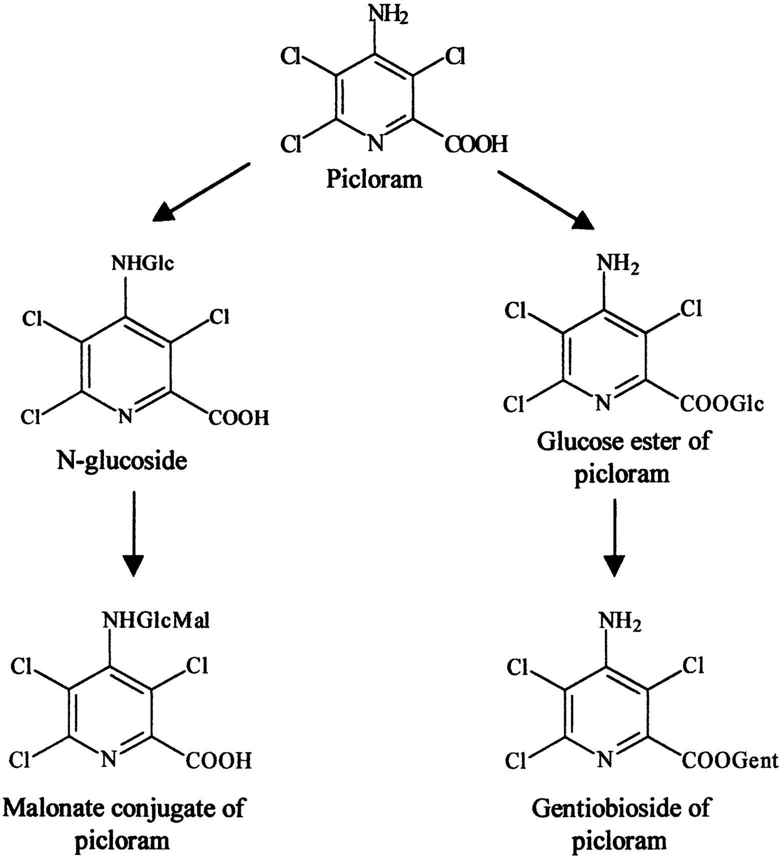

NH2
Cl
Cl
Cl
N
COOH
Picloram
NHGlc
COOH
N-glucoside
COOGlc
Glucose ester of picloram
NHGlcMal
Malonate conjugate of picloram
COOGent
Gentiobioside of picloram

Figure 6. Proposed metabolic scheme for picloram in leafy spurge. Glc = glucoside, Mal = malonate, Gent = gentiobioside. (Adapted from ref 49).

Differential conjugation is believed to affect the tolerance of a plant to a given herbicide. To determine if differential metabolism was responsible for differences in sensitivity to picloram between sunflower (*Helianthus annus*) and rapeseed (*Brassica campestris*), amounts of radiolabelled picloram were measured in the water-soluble fractions of each species (*50*). After nine days, 48% and 78% of the

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch005

recovered picloram was in the water-soluble fraction of sunflower and rapeseed, respectively. The 30% difference in metabolism may account for the difference in sensitivity to picloram between the two species.

Experiments with picloram and clopyralid were conducted to determine if translocation and metabolism were responsible for sensitivity differences between sunflower (susceptible to both picloram and clopyralid) and rapeseed (susceptible to picloram but not clopyralid). Both plants absorbed 97% of applied [1-^{14}C]-picloram and clopyralid, with approximately 60% of the radiolabelled picloram and clopyralid being transported acropetally in both species (*51*). Since more picloram and clopyralid were converted to water-soluble metabolites in rapeseed than in susceptible sunflower, metabolism may account for sensitivity differences between the two species to both herbicides. However, because a significant quantity of applied picloram and clopyralid was not metabolized in rapeseed, differences in sensitivity to these herbicides could not be directly linked to the extent of herbicide metabolism. This difference in sensitivity of rapeseed to picloram and clopyralid was attributed to the difference in sensitivity of the target site to these two herbicides (*51*).

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch005

Case Study – Metribuzin

Background

Metribuzin is an asymmetrical triazine applied either preemergence or early postemergence for control of annual grasses and broadleaf weeds in tolerant crops such as tomato, soybean and wheat. Uptake occurs readily in all plants regardless of their degree of susceptibility or tolerance to the herbicide. Metribuzin translocates with the transpiration stream in the xylem. It also transfers from the xylem to the phloem, however, it quickly returns to the xylem.

The amino group on the fourth carbon of the heterocyclic ring of metribuzin is required for herbicidal activity. Deaminated metribuzin is a major non-phytotoxic metabolite in soybean treated with metribuzin (*52*), but is a relatively minor metabolite in other crops such as tomato. Another detoxification reaction of metribuzin is sulfoxidation of the 3-methylthio group of the heterocyclic ring (*53*). This reaction predisposes metribuzin to subsequent conjugation with homoglutathione in soybean.

Selectivity Differences Among Soybean Cultivars

The major pathway for the metabolism of metribuzin involves conjugation to homoglutathione. A minor pathway of metabolism involves formation of an intermediate *N*-glucoside conjugate (*31*), followed by a second conjugation to yield a malonic acid conjugate.

Two soybean cultivars (Essex and Coker 102) were found to metabolize metribuzin differently (*54*). The ether fraction in the tolerant Essex cultivar contained at least twice the amount of deaminated (inactive) metribuzin than the susceptible Coker 102 cultivar, leading to the conclusion that Essex was more tolerant to metribuzin due to more rapid metabolism of the herbicide to non-phytotoxic products.

Subsequently, it was found that intraspecies differential tolerance to metribuzin could be attributed to the restriction of metribuzin to vascular tissue in tolerant Coker 338 and movement of metribuzin to interveinal tissue in susceptible Semmes (*55*). Metribuzin and its metabolites were characterized as they translocated from the roots to the shoots. Metribuzin was found to be metabolized in the xylem. Since xylem parenchyma cells are very close to the xylem and they have ATPase activity, they may metabolize metribuzin. Once metabolized, the more polar metabolites may be unable to penetrate the parenchyma cell membrane or re-enter the transpiration stream and become compartmentalized in the vascular tissue. Consequently, the water-soluble metabolites were effectively blocked from reaching the leaf chloroplasts. Furthermore, autoradiographs revealed more ^{14}C label in the veins of the tolerant than susceptible cultivar (*56*).

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch005

Metabolism of Metribuzin in Tomato

In tomato, metribuzin is rapidly absorbed, translocated and metabolized into polar products. The major metabolite is an *N*-glucoside, which is subsequently metabolized to UDPG *N*-malonyl-glucoside, whereas a homogluathione conjugate is a minor metabolite (*31*).

No differences were found in the uptake or translocation of metribuzin in various tomato cultivars, however, a 16-fold difference in metribuzin tolerance has been observed between cultivars. This variation in tolerance has been attributed to the differences in metribuzin metabolism via the *N*-glucoside pathway. Varying activity of UDPG *N*-glucosyl transferase on metribuzin or availability of substrates for the reaction could be responsible for the differential tolerance (*57*).

When UDPG *N*-glucosyl transferase activity was measured in leaf tissue from young seedlings of tolerant and sensitive tomato cultivars, it was found that tolerant tomato cultivars had 1.5-fold greater activity than metribuzin-sensitive cultivars. Leaf tissue from older seedlings did not exhibit these differences in enzyme activity (*58*). A differential response was also noted in tomato plants depending on the hours of light the plants received prior to the application of metribuzin (*59*). Reduced light decreased carbohydrate reserves and UDPG substrate levels for *N*-glucoside biosynthesis and ultimately diminished metribuzin detoxification, resulting in their increased sensitivity to the herbicide (*58*).

Conjugation Reactions in Microorganisms

Microorganisms do not have a multifaceted physical barrier like animals and plants (e.g. epidermis or cuticle) to protect them from the external environment. Therefore microorganisms and animals have evolved different detoxification systems and these have been described in the literature (*60*). Conjugation reactions in microorganisms includes xylosylation, alkylation, acylation, and nitrosation. In animals and plants, conjugation reactions are mainly found intracellularly, after absorption of xenobiotics, whereas in microorganisms these reactions can occur both intra- and extracellularly. Microbial conjugation reactions can be distinguished from other types of chemical reactions by covalent binding of a natural metabolite produced by the microorganism with the xenobiotics during metabolism.

Although fungi are less abundant than bacteria in soil, they can penetrate and occupy extensive environmental niches due to their extended mycelial growth pattern. As a result, fungi have evolved metabolic pathways different from bacteria to acquire nutrients and metabolize xenobiotics to prevent exposure to toxic compounds. Most often, bacteria can completely degrade certain xenobiotics such as herbicides to inorganic compounds to produce energy. In contrast, fungi introduce minor chemical changes to the original compound without subsequent metabolism. This type of fungal metabolism is termed biotransformation, a protective action preventing the accumulation of toxic substances in the organisms while conserving metabolic energy (*61, 62*).

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch005

Conjugation Reactions in Fungi

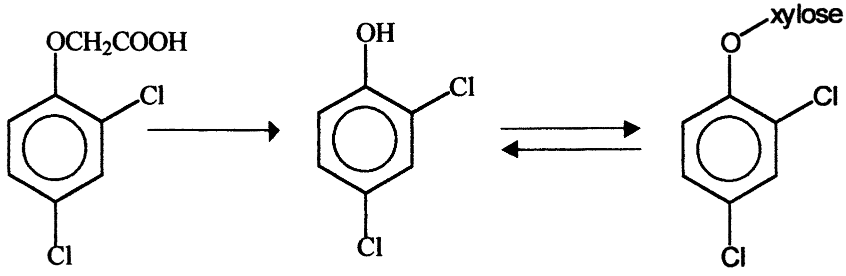
Xylosylation (Xylose Conjugation)

Every year, more than 40 billion tons of carbon is fixed by photosynthesis and incorporated into plants as structural polymers, accounting for roughly 50% of total fixed carbon dioxide on earth. These polymers include lignin and polysaccharides. The former is a non-carbohydrate polymer while the latter includes pectin, cellulose and hemicellulose. Microorganisms that degrade plant polysaccharides and lignin play an important role in the global carbon cycle. Among cellulose-utilizing microorganisms, cellulolytic fungi efficiently depolymerize these polysaccharides into their monomers by a wide variety of extracellular cellulolytic enzymes. Cellulose is converted into cellobiose and glucose, while hemi-cellulose is converted into hexose and pentose, the majority of the pentoses being xylose.

Lignin is the most abundant phenolic-based compound in nature. It is mainly found in woody plants and is highly recalcitrant in the environment. This recalcitrance is mainly due to its lack of repetitive structure and the hydrophobic properties of the polymer. More importantly, lignin metabolites, such as phenolic compounds and their free radicals, are toxic to potential degraders in the soil.

White rot basidiomycetous fungi are primarily responsible for the initial depolymerization of lignin during wood decay. When cultured under ligninolytic conditions, the basidiomycete *Dichomitus squalens* produces manganese-dependent peroxidase (MnP) and laccase (*63*). These extracellular enzymes break down cellulose and hemicellulose into their corresponding sugar molecules, which are utilized for energy production. Concomitantly, lignin is depolymerized into monomers for both carbon and nitrogen assimilation. Phenolic monomers of lignin, which are biologically toxic, are conjugated to a xylose molecule through a β-glycosidic bond. The xylosylated phenols become more water-soluble and less toxic to the fungal cells (*64*).

The ability of *D. squalens* to conjugate phenolic compounds from lignin degradation allows transformation of chlorinated phenoxyacetic acid during ligninolytic conditions. The first step in 2,4-D and 2,4,5-T degradation is the extracellular, fungal enzymatic cleavage of the ether linkage, which releases the carbon side chain and chlorinated phenol. The two-carbon side chain of the herbicide is mineralized to CO_2 while the chlorinated phenol is xylosylated extracellularly in a nitrogen-sufficient medium that contains 30 μM Mn^{2+} (Figure 7). The xyloside can be hydrolyzed by a glycosidase to regenerate the chlorophenol, which in turn undergoes oxidative dechlorination catalyzed by MnP with subsequent ring cleavage (*65*). Enzymes catalyzing the ether cleavage, xylosylation and xyloside hydrolysis have not been identified, but it is known that they are involved in secondary metabolism by the extracellular lignin-degrading system (*64*). With an ample supply of sugar monomers from the breakdown of cellulose and hemicellulose, xylose conjugation of phenols appears to conserve metabolic energy in detoxification of the compound by the fungi.



2,4-D

2,4-Dichlorophenol

Xylosylated 2,4-dichlorophenol

Figure 7. Xylosation of 2,4-D by Dichomitus squalens (Adapted from ref 64).

Methylation

Another type of conjugation mechanism found in fungi is methylation. Methylation of organic and inorganic compounds has been studied extensively (*61, 66*). Most of these reactions were studied in animals and plants. Volatile arsenic compounds were first observed to be produced from several fungi growing on inorganic media containing arsenic (*67*), which was subsequently identified as

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch005

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch005

trimethylarsine (*66*). More methylation reactions have since been described, with nitrogenous and phenolic-hydroxyl groups as major target molecules. Definitive reports on methylation of organic pesticides by fungi were not published until the 1970's. Pentachlorophenol, a substance with fungicidal and various other pesticidal properties, was methylated to pentachloroanisole in fungal cultures of *Trichoderma virgatum* (*68*) (Figure 8). Pentachloroanisole is believed to be more resistant to further chemical and biological degradation but is less toxic towards various organisms than the parent chlorinated phenol (*61*).

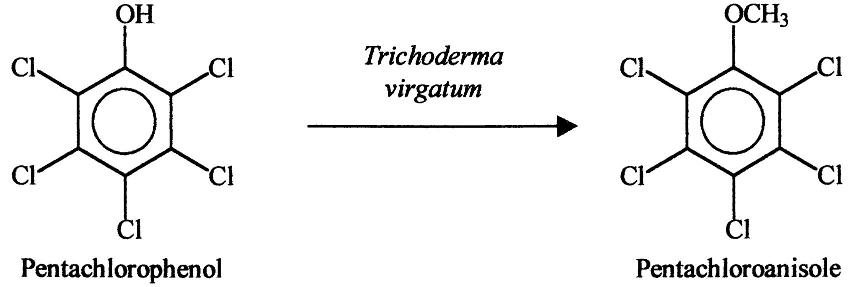


Figure 8. O-methylation of pentachlorophenol by fungal cultures of Trichoderma virgatum (Adapted from ref 65, 66).

Phanaerochaete chrysosporium, the most studied white-rot basidiomycete, produces two types of extracellular heme peroxidases – manganese peroxidase (MnP) and lignin peroxidase (LiP), which, together with an H_2O_2-generating system, constitute the major extracellular components of its lignin-degrading system (*69, 70*). *P. chrysosporium* methylates chlorinated phenols, which are intermediates of chlorophenoxyacetic acid mineralization, to form corresponding anisoles (*65, 71, 72*). It was observed that *P. chrysosporium* exhibited slower rate of chlorophenoxyacetic acid mineralization than *D. squalens* (*64*). This can be partly explained by the fact that methylated phenols are metabolically more stable and less readily to be hydrolyzed to produce their parent compounds for further mineralization than xylosylated phenols found with *D. squalens*.

Methytransferase mediated methyl conjugation to a thiol group of xenobiotics occurs in *Saccharomycopsis lipolytica* (*73*). In this yeast, the fungicide pentachloronitrobenzene was first converted by glutathione-S-transferase to the thiol-derivative, then methylated by the action of S-adenosylmethionine-dependent thiol methyltransferase and excreted out of the cell. Detectable amounts of the methylthiol derivative of the fungicide accumulate extracellularly, but the excretion mechanism is unknown. Generally, methylthiol derivatives are less toxic than their parent thiol-benzenes. However, further processing of methylthiol derivatives may produce toxic products. For example, methylsulfone derivatives of some chlorinated biphenyls are more toxic than their precursors (*73*).

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch005

Another example of methyl conjugation is the transformation of oxadiazon by *Fusarium solani*. Oxadiazon is a preemergent soil-applied herbicide used for the control of annual grasses and broadleaf weeds in rice, soybean, cotton, onion (*Allium cepa*), peanut, potato (*Solanium tuberosum*), and ornamentals. Phenolic, carboxylic acid, and dealkylated derivatives of oxadiazon were identified in soil, but there was no evidence of aromatic ring cleavage or the presence of dechlorinated products (*74*). It was shown that degradation of the herbicide was a co-metabolic process (*75*). With no dextrose in the culture medium, more than 70% of the applied herbicide remained unchanged after 3 weeks of incubation. In the presence of dextrose, *F. solani* transformed more than 58% of oxadiazon within 3 weeks. These results indicate that *F. solani* cannot effectively utilize oxidiazon as a sole carbon source and that the mechanism of transformation of this herbicide was co-metabolic. The oxadiazoline heterocyclic ring of this herbicide is first cleaved and the intermediate was methylated or decarboxylated before being removed from the aromatic moiety (Figure 9).

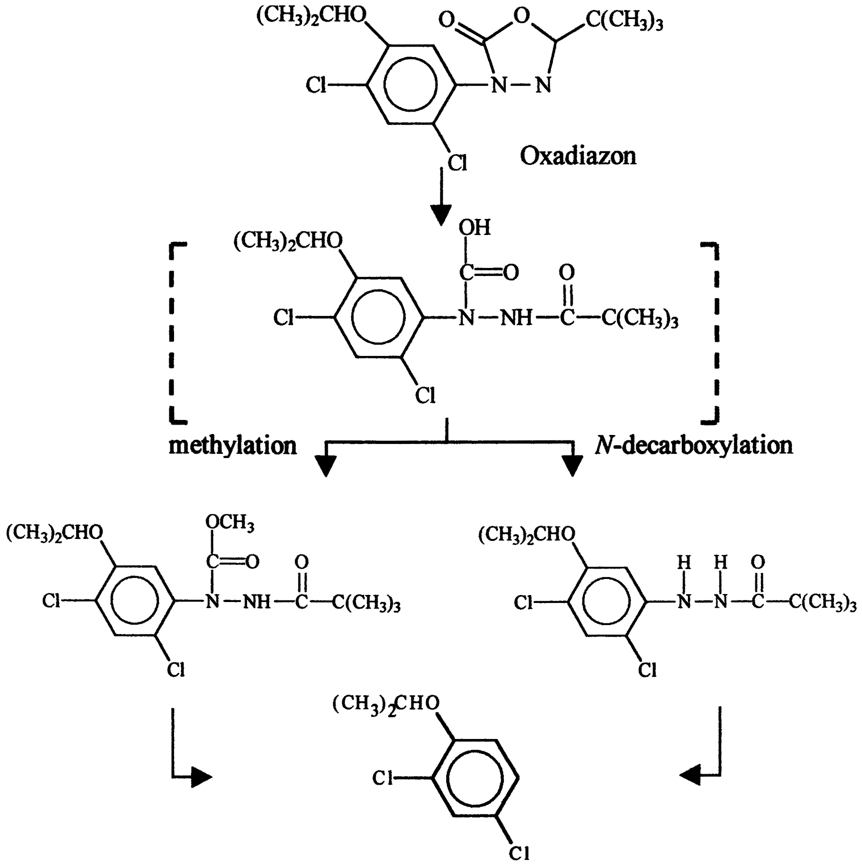


Figure 9. Metabolism of oxadiazon by Fusarium solani (Adapted from ref 75).

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch005

Acylation

Xenobiotics can also be conjugated to acetate and formate by fungi in a process called acylation. This type of conjugation is frequently observed with anilines and phenols in the soil (*60*). Although these compounds are not commonly found in the environment or used directly as pesticides, anilines and phenols are intermediates that are formed during the breakdown of several important groups of herbicides including the phenylacylanilides, phenylcarbamates, and substituted phenylureas (*61*). These intermediates are still biologically active and may undergo further transformation reactions.

Metobromuron is a preemergence phenylurea herbicide used primarily on beans (*Phaseolus* spp.), potato, soybean, tobacco and tomato. In the soil, metobromuron is quickly hydrolysed at the urea bridge to produce 4-bromoaniline, which is further acetylated to 4-bromoacetanilide (Figure 10) by pure fungal cultures of *Talaromyces wortmanni*, *Fusarium oxysporum*, bacterial cultures of *Bacillus* sp. and the green algae *Chorella vulgaris*. However, 4-bromoaniline was never isolated from the cultures when metobromuron was used as a substrate. Due to the lack of accumulation of 4-bromoaniline, it was hypothesized that acetylation of 4-bromoaniline was rapid (*61*).

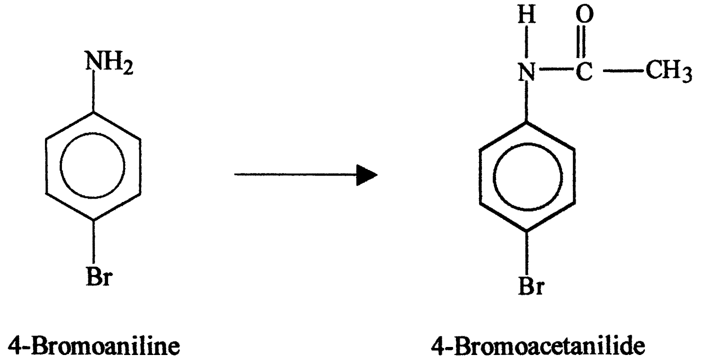


Figure 10. Acetylation of 4-bromoaniline by fungal cultures of Talaromyces wortmanni and Fusarium oxysporum. (Adapted from ref 76.)

Chlorinated anilines are also conjugated to amino acids during their mineralization by *P. chrysosporium* (*77*). In a carbon and nitrogen rich medium, the amino group of 3,4-dichloroaniline is first conjugated to α-ketoglutaryl~S-CoA forming *N*-(3,4-dichlorophenyl)-α-ketoglutaric acid. Since this α-ketoglutaric acid conjugate is unstable, it is self-cyclized prior to further degradation.

Significance of Xenobiotic Conjugation by Fungi

The identification of acetyl conjugation in fungi led to the development of a pollutant decontamination strategy based on the coupling of herbicides to soil material. The conjugation of anilines to form *N*-acetyl derivatives competed with the oxidative self-coupling reactions of anilines in soil (*61*). These coupling reactions led Bollag and coworkers (*78*) to investigate potential bioremediation alternatives for pollutants in soil. During the continuous humification process in soil, certain pesticides or their degradation products, that are structurally similar to the humic acid precursors, bind to the soil and become unavailable to living organisms. For this reason, researchers may be able to replace invasive approaches, e.g. applying detergents, to decontaminate certain polluted sites with indigenous microorganisms.

Bentazon can be hydroxylated and incorporated into soil humic substances (*78*). It is bound to the soil matrix, specifically to humic monomers, including ferulic acid and guaiacol. The binding rate is increased in the presence of laccase extracted from the fungus *Polyporus pinsitus*. An oxidation reaction catalyzed by laccase is proposed as the first step in the coupling reaction in soil. Susceptible substrates, including most of the humic monomers, namely catechol, ferulic acid and guaiacol, are oxidized by removal of a hydrogen atom from their hydroxyl group (Figure 11). The free radical that is formed is very unstable and reactive to any nucleophilic moieties such as NH_2 (*78, 79, 80*). Bentazon or its metabolites are coupled to the oxidized products of humic monomers with loss of their phytotoxicity. In addition, these coupled complexes are no longer biologically available to living organisms.

Some pesticides, particularly when they are partially degraded, yield phenol- or aniline-like chemicals analogous to naturally occurring compounds. Microbial phenoloxidases and peroxidases catalyze the polymerization of these pesticide metabolites (*81*). Laccase, isolated from the soil fungus *Rhizoctonia praticola,* oxidatively coupled halogenated phenols that originated from phenoxyalkanoate herbicides, to form dimers, trimers, and tetramers. These condensation reactions may have profound effects on the bioremediation of contaminated soil sites. Phenol and/or aniline derivatives of pesticides may be immobilized by inoculating fungal cultures or by enhancing growth and activity of indigenous fungi in the environment (*79, 82, 83, 84*).

Bacterial Conjugation Reactions

Methylation

Methylation reactions are usually described in bacteria in reference to metabolism of metals or metalloid pesticides, such as mercury and arsenic (*60*). The use of mercury in herbicides has been phased out, but organic arsenical compounds, such as monosodium methylarsonate (MSMA) and cacodylic acid, are still used as herbicides throughout the world.

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch005

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch005

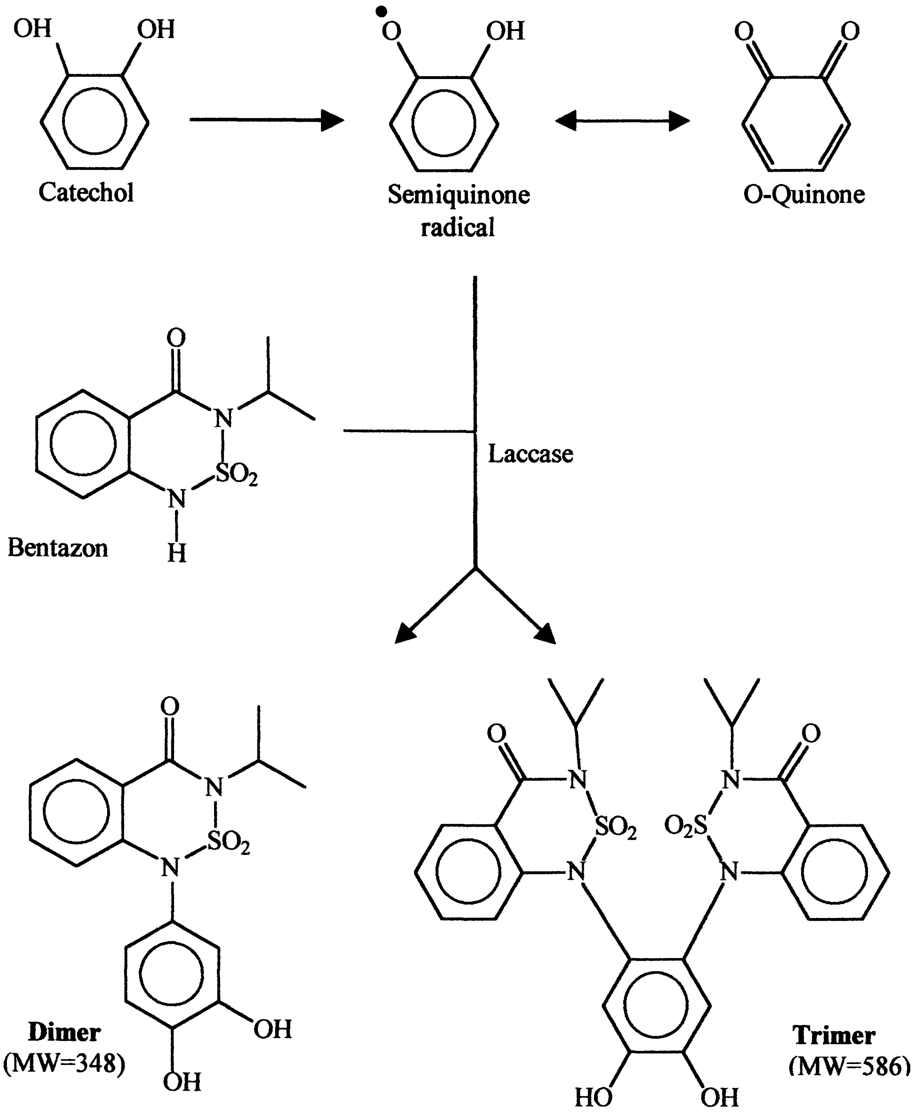

Catechol
Semiquinone radical
O-Quinone
Bentazon
Laccase
Dimer
(MW=348)
Trimer
(MW=586)

Figure 11. Humification of bentazon catalyzed by laccase of Polyporus pinsitus (Adapted from ref 82).

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch005

The methylation of divalent mercury to methyl mercury occurs in biological systems. The methyl group is transferred from methyl-cobalamin to Hg^{2+} by an anaerobic bacterium isolated from water sediment (*85*). Methyl-cobalamin, a methyl-carrier, is the substrate required for the formation of methane in methanogenesis. However, at low Hg^{2+} concentration, methane production is strongly inhibited, while methyl- and dimethyl mercury are produced. Methylated mercury products have a high potential for bioaccumulation in the aquatic environment. The toxicity of methylated mercury compounds to humans and animals has resulted in the restriction and banning of certain mercury containing pesticides.

In contrast to mercury, arsenic compounds are methylated by the Challenger mechanism, named in honour of the late Frederick Challenger who first proposed this mechanism (*86*). S-adenosylmethionine (SAM) is the methylating agent that transfers the methyl group as a carbo-cationic intermediate (CH_3^+). Any recipient atom must be nucleophilic. Arsenate (+5) is first reduced to arsenic acid (-3) in which the non-bonded electron pair is electrophilically attacked by SAM. A toxic but relatively unstable methanoarsonic acid is formed and further methylated to dimethylarsinic acid and finally reduced to dimethylarsine (Figure 12). *Methanobacterium* sp. and *Scopulariopsis brevicaulis* can directly reduce cacodylic acid to dimethylarsine (*60*).

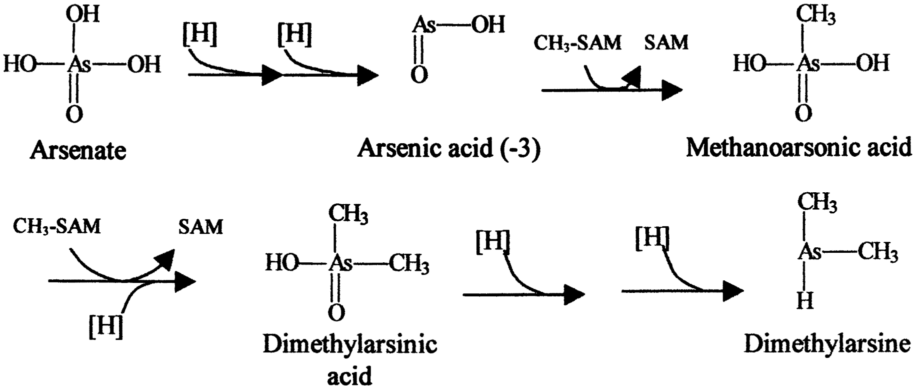


Figure 12. Transformation of arsenic by gram-positive and –negative bacteria. (e.g. Acinetobacter sp. and Rhodococcus sp.)(Adapted from ref 60).

The ability to *O*-methylate hydroxylated organic compounds is widespread in soil bacteria and a detailed review on *O*-methylation of chlorinated monoaromatic compounds by soil bacteria is available (*87*). *O*-methylation is an environmentally important conjugation reaction of organic compounds and this reaction increases their lipophilicity and, therefore, increases the potential for bioaccumulation of transformed products. Chloroanisoles are produced by soil bacteria incubated with chlorophenols or chlorophenoxyacetic acids, similar to fungal conjugation reactions described in the previous section (Figure 8).

Several species of gram-negative bacteria belonging to the genera *Acinetobacter* and *Pseudomonas*, and gram-positive bacteria belonging to the genera *Rhodococcus* and *Mycobacterium* have been shown to *O*-methylate chlorinated phenols and phenol derivatives (*87*). These organisms are widely distributed in nature and, therefore, play an important role in both the degradation and transformation of chlorophenols in the environment. Researchers using cell extracts from both gram-positive and -negative bacteria have demonstrated that the *in vivo* methyl donor is SAM.

The enzyme responsible for the methyl transfer has not been characterized. However, a wide range of halogenated phenols and phenol derivatives can be *O*-methylated indicating that the enzyme has a wide substrate range (*88*). *Rhodococcus sp*. methylated several substrates, each at a different rate (*89*). Dihydroxy compounds, such as tetrachlorocatechol and tetrachloro-hydroquinone, are better substrates than chlorophenols and are methylated to corresponding mono- and di-methoxy compounds. The sulfhydryl group of chlorothiophenols is methylated 10-fold faster than the hydroxyl group of chlorophenols. On the other hand, the same bacteria do not methylate the amino group of pentachloroaniline. In addition, the enzymatic activity was not influenced by the nature of the growth substrate, suggesting that *O*-methylating activity was constitutive (*90*).

Nitrosation

Nitrosation is another conjugation reaction observed in bacteria in which a nitrite is conjugated to a secondary amine to form nitrosamine (*91*). Some bacterial species appear to enzymatically conjugate a nitrite group to a secondary amine, whereas in *Pseudomonas stutzeri*, nitrosamines are generated in a non-enzymatic process involving cell constituents (*91*). Nitrosamines represent a class of compounds that are extremely hazardous to mammals. Members of this group of compounds are carcinogenic, mutagenic and teratogenic. Exposure of individuals to these compounds may occur through the consumption of crops that have assimilated the toxicant from the environment or from water containing the *N*-nitroso compound as a result of leaching (*92*).

S-triazine herbicides are powerful inhibitors of photosynthetic electron flow in plants (*93*). They interrupt the light-driven flow of electrons from water to nicotinamide adenine dinucleotide phosphate ($NADP^+$), energy transfer, and ultimately inhibit carbon fixation. Most herbicides in this class contain one or two secondary amine groups. In the soil, under nitrifying conditions, nitrate serves as electron acceptor during nitrification and is reduced to nitrite by nitrifying bacteria at alkaline pH in soil. The nitrites formed can conjugate to secondary amine groups of the herbicide and produce the nitrosoamines. Studies on identification of nitrosamine derivatives of s-triazines have not been reported, but due to their

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch005

hazardous nature, we believe investigations on their potential presence in the environment should be undertaken.

Glutathione Conjugation

Glutathione (GSH) was first found in cyanobacteria and purple bacteria. This finding led to the hypothesis that glutathione was produced to protect the cells from oxygen stress. High levels of glutathione were later found in anaerobic green sulfur bacteria and provided evidence that this compound is also involved in anaerobic sulfide metabolism (*94*).

Glutathione *S*-transferases (GSTs), which are also found in plants and animals, are one of the most extensively studied glutathione-dependent enzymes in microorganisms. Members in this enzyme family catalyze the conjuation of the bioactive thiol – GSH to electrophilic substrates. Similar to the GSTs found in plants described earlier in this chapter, bacterial GSTs have been shown to detoxify various xenobiotics through conjugation with GSH (*95, 96*). Isolates from the rhizosphere are able to detoxify herbicides of two different families, chloroacetamides and diphenyl ethers (*97, 98*). The herbicide-GSH conjugates are subsequently metabolized to cysteine conjugates, which are transformed to the corresponding thiols, pyruvate, and ammonia by cysteine β-lyases (*99*). In addition, soil bacteria are able to oxidize thiols to sulfonates. Sulfonated metabolites of alachlor and metolachlor have been detected and identified in groundwater and soil (*100, 101*). These results illustrated the potential of using soil bacteria for various bioremediation strategies. Although the isolated degrading bacteria are less competitive upon reintroduction to the environment, the combination of molecular biological and protein chemical techniques (*99*) available today may result in better efficiency and control of bioremediation using bacteria. A review of bacterial GSTs with regard to xenobiotic degradation is also presented in a separate chapter of this book.

Conclusion

The types of conjugation reactions that occur in plants and soil microorganisms (intra- and extracellularly), are dependent on the availability of different types of endogenous compounds. Since carbohydrates are readily available in plants during photosynthesis, these compounds are most commonly involved in conjugation reactions. On the other hand, most microorganisms are heterotrophs that require an external supply of nutrients. It is metabolically too expensive for microbes to use the limited source of carbohydrates and other nutrients for xenobiotic transformation. Most conjugation reactions of xenobiotics in microorganisms involve intermediates from primary metabolism. The resulting conjugates become less toxic to the organism, and/or are chemically excluded from metabolic processes. In plants, the exclusion is physical, conjugates are compartmentalized into vacuoles, cell wall lignin and vascular tissues, thus preventing many phytotoxic effects.

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch005

There are also some similarities between mechanisms found in plants and microorganisms. Both groups of organisms can detoxify xenobiotics using co-metabolism; where existing enzymatic machinery is adapted to aid in the detoxification process. There are several types of conjugation reactions that are common to both groups. For example, conjugation to glutathione is an important detoxification reaction in both plants and microbes. Acylation and methylation reactions also occur in both organisms. However, glucosides are the most common conjugate in plants, but only one example of glycosylation has been found in fungi: the xylosation of a chlorinated phenol.

In microbes, conjugation occurs to aid transformation of the xenobiotics by using existing metabolic pathways to detoxify the xenobiotics. Herbicides in the soil can be immobilized by conjugation reactions and become biologically unavailable. The same principle applies for the decontamination of pesticides in soil by conjugating a pesticide or its metabolites to soil humic substances (*78*). However, soil microbes can conjugate certain pesticides, which makes them more lipophilic and persistent in the environment. These metabolites may have potential to bioaccumulate in the food chain. Similarly, in plants, certain amino acid conjugates of 2,4-D are less polar than 2,4-D, thereby maintaining their phytotoxic properties.

Conjugation reactions constitute part of the transformation mechanisms found in living organisms. In plants, different crops have become resistant to herbicides by expressing high levels of an enzyme responsible for converting the herbicide into non-toxic metabolites (*58*). Crop or weed species that are susceptible to a certain herbicide may have lower levels of a specific enzyme required for detoxification to occur via conjugation. Also, pesticides with two or more functional groups can form a range of conjugates. Glucose esters of chloramben or 2,4-D are unstable in the cytoplasm and are metabolized to the parent herbicide. Other conjugates, such as *N*-glucosides are more stable. This difference in stability can confer tolerance to the crop that preferentially conjugates the herbicide to *N*-glucosides (*18*).

Many of the enzymes responsible for glucose conjugation have not been studied in detail, nor have they been found in all plants that produce glucose conjugates. Although amino acid conjugation in plants may be an enzymatic process, no enzymes have yet been characterized. Information on enzymatic properties and their reaction mechanisms in conjugation reactions is lacking, and additional research is required to learn more about the biochemistry and physiology of herbicide metabolism. Recent advances in recombinant DNA technology and biotechnology will allow for the expression of microbial enzymes catalyzing conjugation reactions that may confer pesticide resistance in transgenic crops.

Based on our discussion of conjugation, we believe that Dorough's definition of conjugation (*3*) does not completely describe the diversity of conjugation reactions found in plants and microorganisms. Conjugation reactions found in, or mediated by, microorganisms (external to microbes) do not always produce conjugates with higher water solubility, but are essential for continuous metabolism of the pesticides. For these reasons, an extension of the definition of a conjugation reaction is proposed. "Conjugation is a metabolic process whereby an exogenous or

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch005

endogenous natural compound is joined to a pesticide or its metabolite(s) facilitating compartmentalization, sequestration, detoxification and/or mineralization." We believe our definition describes how conjugation of pesticides occurs in both plants and microorganisms.

References

1. Pimental, D.; Levitan, L. *Biosciences.* **1986**, *36*, 86-91.
2. Wolfe, M.F.; Seiber, J.N. *Occupational Medicine.* **1993**, *8*, 561-573.
3. Dorough, H.W. In *Bound and Conjugated Pesticide Residues*; Kaufman, D.D.; Still, G.G.; Paulson, G.D.; Bandal, S.K., Eds.; American Chemical Symposium Series 29; ACS: Wash., D.C., 1976; pp. 11-34.
4. Alexander, M. *Microbiol. Rev.* **1979**, *43*, 95-102.
5. Geissbühler, H.; Martin, H.; Voss, G. In *Herbicides: Chemistry, Degradation, and Mode of Action*; Kearney, P.C.; Kaufman, D.D., Eds.; Marcel Dekker, Inc.: New York, NY, 1975; Vol. 1, pp. 209-291.
6. Feung, C.; Mumma, R.O.; Hamilton, R.H. *J. Agric. Food Chem.* **1974**, *22*, 307-309.
7. Shimabukuro, R.H. In *Weed Physiology Volume II: Herbicide Physiology*; Duke, S.O. Ed.; CRC Press, Inc.: Boca Raton, Fl, 1985; pp. 215-240.
8. Hatzios, K.K. In *Environmental Chemistry of Herbicides;* Grover, R.; Fich, M.; CRC Press: Boca Raton, FL, 1991; Vol 2, pp. 141-185.
9. Kreuz, K.; Tommasini, R.; Martinoia, E. *Plant Physiol.* **1996**, *111*, 349-353.
10. Kreuz, K. Abstracts Weed Sci. Soc. Am. 1999 Meeting. pp 178.
11. Swanson, C.R.; Kadunce, R.E.; Hodgson, R.H.; Frear, D.S. *Weeds.* **1966**, *14*, 319-323.
12. Kaslander, J.; Sijesteijn, A.K.; Van der Kerk, G.J.M. *Biochim. Biophys. Acta.* **1961**, *52*, 396-397.
13. Aritomi, M; Kawasaki, T. *Phytochemistry.* **1984**, *23*, 2043-2047.
14. Lamoureux, G.L.; Rusness, D.G. In *Xenobiotic Conjugation Chemistry*; Paulson, G.D.; Caldwell, J.; Hutson, D.H.; Menn, J.J., Eds.; American Chemical Society Symposium Series 299. ACS: Wash., D.C., 1986; pp. 62-105.
15. Mine, A.; Miyakado, M.; Matsunaka, S. *Pestic. Biochem. Physiol.* **1975**, *5*, 566-574.
16. Edwards, V.T.; McMinn, A.L.; Wright A.N. In *Progress in Pesticide Biochemistry*; Hutson, D.H.; Roberts, T.R., Eds.; John Wiley and Sons, Ltd: New York, NY, 1982; Vol. 2, pp 71-125.
17. Dennis, D.T.; Turpin, D.H.; Lefebvre, D.D.; Layzell, D.B. *Plant Metabolism* 2nd Ed. Addison Wesley Longman Limited: Essex, U.K., 1997; pp. 89-157.
18. Frear, D.S.; Swanson, H.R.; Mansager, E.R.; Wien, R.G. *J. Agric. Food Chem.* **1978**, *26*, 1347-1351.

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch005

19. Hodgson, R.H.; Frear, D.S.; Swanson, H.R.; Regan, C.A. *Weed Sci.* **1973**, *21*, 542-549.
20. More, J.E.; Roberts, T.R.; Wright, A.N. *Pestic. Biochem. Physiol.* **1978**, *9*, 268-280.
21. Frear, D.S. In *Bound and Conjugated Pesticide Residues*; Kaufman, D.D.; Still, G.G.; Paulson, G.D.; Bandal, S.K., Eds.; American Chemical Symposium Series 29. ACS: Wash., D.C. 1976; pp 35-54.
22. Klambt, H.D. *Planta.* **1961**, *57*, 339-353.
23. Sandermann, H. Jr.; Haas, M.; Messner, B.; Pflumacher, S.; Schröder, P.; Wetzel, A. In *Regulation of Enzymatic Systems Detoxifying Xenobiotics in Plants* NATO ASI Series Vol 39; Hatzios, K.K. Ed.; Kluwer Academic Publishers: Norwell, MA, 1997; pp. 211-231.
24. Shimabukuro, R.H.; Lamoureux, G.L.; Swanson, H.R.; Walsh, W.C.; Stafford, L.E.; Frear, D.S. *Pestic. Biochem. Physiol.* **1973**, *3*, 483-494.
25. Hatzios, K.K.; Penner, D. *Metabolism of Herbicides in Higher Plants*; Burgess Publishing Co.: Minneapolis, MN, 1982; pp 43-58.
26. Marrs, K.A. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1996**, 47, 127-158.
27. Cole, D.J.; Cummins, P.J.; Hutton, P.J.; Dixon, D.; Edwards, R. In *Regulation of Enzymatic Systems Detoxifying Xenobiotics in Plants,* NATO ASI Series, Vol 39; Hatzios, K.K. Ed.; Kluwer Academic Publishers: Norwell, MA, 1997; pp. 139-154.
28. Jakoby, W.B.; Keen, J.H. *Trends Biochem. Sci.* **1977**, *2*, 229-231.
29. Devine, M.D.; Duke, S.O.; Fedtke, C. *Physiology of Herbicide Action*; Prentice Hall Inc.: Englewood Cliffs, NJ, 1993.
30. Tal, J.A.; Hall, J.C.; Stephenson, G.R. *Weed Res.* **1995**, *35*, 133-139.
31. Frear, D.S.; Swanson, H.R.; Mansager, E.R. *Pestic. Biochem. Physiol.* **1985**, *23*, 56-65.
32. Martinoia, E.; Grill, E.; Tommasini, R.; Kreuz, K.; Amrhein, N. *Nature.* **1993**, *364*, 247-249.
33. Coleman, J.O.D.; Randall, R.; Blake-Kalff, M.M.A. *Plant, Cell and Environ.* **1997**, *20*, 449-460.
34. Feung, C.; Hamilton, R.H.; Witham, F.H. *J. Agric. Food Chem.* **1971**, *19*, 475-479.
35. Andreae, W.A.; Good, N.E. *Plant Physiol.* **1957**, 32, 566-572.
36. Feung, C.; Hamilton, R.H.; Mumma, R.O. *J. Agric. Food Chem.* **1973**, *21*, 637-640.
37. Feung, C.; Hamilton, R.H.; Witham, F.H.; Mumma, R.O. *Plant Physiol.* **1972**, *50*, 80-86.
38. Parkinson, A. In *Casarett and Doull's Toxicology 5th Edition*; Klaassen, C.D., Ed.; McGraw-Hill Inc: New York, NY, 1996; pp. 113-186.
39. Mumma, R.O.; Hamilton, R.H. In *Bound and Conjugated Pesticide Residues*; Kaufman, D.D.; Still, G.G.; Paulson, G.D.; Bandal, S.K., Eds.; American Chemical Symposium Series 29. ACS: Wash., D.C. 1976; pp 68-85.

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch005

40. Feung, C.; Loerch, S.L.; Hamilton, R.H.; Mumma, R.O. *J. Agric. Food Chem.* **1978**, *26*, 1064-1067.
41. Feung, C.; Hamilton, R.H.; Mumma, R.O. *J. Agric. Food Chem.* **1975**, *23*, 373-376.
42. Feung, C.; Hamilton, R.H.; Mumma, R.O. *J. Agric. Food Chem.* **1977**, *25*, 898-900.
43. Davidonis, G.H.; Hamilton, R.H.; Mumma, R.O. *Plant Physiol.* **1980**, *66*, 537-540.
44. Montague, M.J.; Enns, R.K.; Siegel, N.R.; Jaworski, E.G. *Plant Physiol.* **1981**, *67*, 603-607.
45. Butcher, H.C.; Wagner, G.J.; Siegelman, H.W. *Plant Physiol.* **1977**, *59*, 1098-1103.
46. Schmitt, R.; Sandermann, H. Jr. *Zeit. Naturforsch.* **1982**, *37c*, 772-777.
47. Still, G.G.; Balba, H.M.; Mansager, E.R. *J. Agric. Food Chem.* **1981**, *29*, 739-746.
48. Scheel, D.; Sandermann, H. Jr. *Planta.* **1981**, *152*, 253-258.
49. Frear, D.S.; Swanson, H.R.; Mansager, E.R. *J. Agric. Food Chem.* **1989**, *37*, 1408-1412.
50. Hallmen, U. *Physiol. Plant.* **1975**, *34*, 266-272.
51. Hall, J.C.; Vanden Born, W.H. *Weed Sci.* **1988**, *36*, 9-14.
52. Fedtke, C. *Pestic. Sci.* **1986**, *17*, 65-66.
53. Trebst, A.; Wietoska, H. *Zeit. Naturforsch.* **1975**, *30c*, 499-504.
54. Mangeot, B.L.; Slife, F.E.; Rieck, C.E. *Weed Sci.* **1979**, *27*, 267-269.
55. Falb, L.N.; Smith, A.E. *J. Agric. Food Chem.* **1984**, *32*, 1425-1428.
56. Falb, L.N.; Smith, A.E. *Pestic. Biochem. Physiol.* **1987**, *27*, 165-172.
57. Smith, A.E.; Phatak, S.C.; Emmatty, D.A. *Pestic. Biochem. Physiol.* **1989**, *35*, 284-290.
58. Frear, D.S.; Mansager, E.R.; Swanson, H.R.; Tanaka, F.S. *Pestic. Biochem. Physiol.* **1983**, *19*, 270-281.
59. da Silva, J.F.; Warren, G.F. *Weed Sci.* **1976**, *24*, 612-615.
60. Kaufman, D.D.; Kearney, P.C. In *Herbicides: Physiology, Biochemistry, Ecology*; Audus, L.J., Ed.; Academic Press: London, UK, 1976; pp. 29-64.
61. Bollag, J.M. *CRC Crit. Rev. Microbiol.* **1972**, *2*(1), 35-58.
62. Cerniglia, C.E. *Biodegradation.* **1992**, *3*, 351-368.
63. Périé, F.H.; Sheng, D.; Gold, M.H. *Biochim. Biophys. Acta.* **1996**, *1297*, 139-148.
64. Reddy, G.V.B.; Joshi, D.K.; Gold, M.H. *Microbiology.* **1997**, *143*, 2352-2360.
65. Joshi, D.K.; Gold, M.H. *Appl. Environ. Microbiol.* **1993**, *59*, 1779-1785.
66. Iwan, J. In *Bound and Conjugated Pesticide Residues*; Kaufman, D.D.; Still, G.G.; Paulson, G.D.; Bandal, S.K., Eds.; ACS Symposium Series 29, ACS: Wash., D.C. 1976; pp 132-152.
67. Williams, R.T. *Detoxicfication Mechanisms* 2nd Ed.; Chapman and Hall, Ltd.: London, UK, 1959; pp 1-256.
68. Cserjesi, A.J.; Johnson, E.L. *Can. J. Microbiol.* **1972**, *18*, 45-50.

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch005

69. Ericksson, K-E.L.; Blanchette, R.A.; Ander, P. In *Microbial and Enzymatic Degradation of Wood and Wood Components*; Timell, T.E., Ed.; Springer-Verlag: Berlin, NY, 1990; pp. 225-333.
70. Gold, M.H.; Alic, M.A. *Microbiol. Rev.* **1993**, *57,* 605-622.
71. Lamar, R.T.; Dietrich, D.M. *Appl. Environ. Microbiol.* **1990**, *56*, 3093-3100.
72. Valli, K.; Gold, M.H. *J. Bacteriol.* **1991**, *173*, 345-352.
73. Drotar, A-M.; Fall, R. *Experientia.* **1985**, *41,* 762-764.
74. Ambrosi, D.; Kearny, P.C.; Macchia, J.A. *J. Agric. Food Chem.* **1977**, *25*, 868-872.
75. Chakraborty, S.K.; Chowdhury, A.; Bhattacharyya, A.; Ghosh, S.; Pan, S.; Waters, R.; Adityachaudhury, N. *J. Agric. Food Chem.* **1995**, *43*, 2964-2969.
76. Tweedy, B.G.; Loeppky, C.; Rhodes, R.C. *Science.* **1970**, *168*, 482-483.
77. Sandermann, H. Jr.; Heller, W.; Hertkorn, N.; Hoque, E.; Pieper, D.; Winkler, R. *Appl. Environ. Microbiol.* **1998**, *64,* 3305-3312.
78. Kim, J.E.; Wang, C-J.J.; Bollag, J.M. *Biodegradation.* **1998**, *8*, 387-392.
79. Bollag, J.M.; Myers, C.J.; Pal, S.; Huang, P.M. In *Environmental Impact of Soil Component Interactions*; Huang, P.M.; Berthelin, J.; Bollag, J.M.; McGill, W.B.; Page, A.L., Eds.; CRC Press, Inc.: Boca Raton, Fl, 1995; pp 299-309.
80. Kim, J.E.; Fernandes, E.; Bollag, J.M. *Environ. Sci. Technol.* **1997**, *31*, 2392-2398.
81. Bollag, J.M. In *Microbial Transformations of Bioactive Compounds*; Rosazza, J.P., Ed.; CRC Press Inc.: Boca Raton, Fl, 1982; Vol. 2, pp 126-168.
82. Dec, J.; Bollag, J.M. *Arch. Environ. Contamin. Toxicol.* **1990**, *19*, 543-550.
83. Dec, J.; Bollag, J.M. *Soil Sci.* **1997**, *162*, 858-874.
84. Dec, J.; Haider, K.; Rangaswamy, V.; Schäffer, A; Fernande, E.; Bollag, J.M. *J. Agric. Food Chem.* **1997**, *45*, 514-520.
85. Wood, J.M.; Kennedy, F.S.; Rosen, C.G. *Nature.* **1968**, *220*, 173-174.
86. Challenger, F. In *Organometals and Organometalloids: Occurrence and Fate in the Environment*; Brinkman, F.E.; Bellama, J.M., Eds.; ACS Books: Wash., D.C., 1978; pp 1-20.
87. Häggblom, M.M. *J. Basic Microbiol.* **1990**, *2*, 115-141.
88. Häggblom, M.M.; Nohynek, L.J.; Salkinoja-Salonen, M.S. *Appl. Environ. Microbiol.* **1988**, *54*, 3043-3052.
89. Neilson, A.H.; Lindgren, C.; Hynning, P.-A.; Remberger, M. *Appl. Environ. Microbiol.* **1988**, *54*, 524-530.
90. Suzuki, T.J. *Pestic. Sci.* **1983**, *8*, 419-428.
91. Mills, A.L.; Alexander, M. *Appl. Environ. Microbiol.* **1976**, *31*, 892-895.
92. Ayanaba, A.; Verstraete, W.; Alexander, M. *Soil Sci. Soc. Amer. Proc.* **1973**, 565-568.
93. Esser, H.O.; Marco, G.J. In *Herbicides: Chemistry, Degradation, and Mode of Action*; Kearney, P.C.; Kaufman, D.D., Eds.; Marcel Dekker, Inc.: New York, NY, 1975; Vol. 1, pp 129-208.
94. Bartsch, R.G.; Newton, G.L.; Sherril, C.; Fahey, R.C. *J. Bacteriol.* **1996**, *178*, 4742-4746.

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch005

95. Lau, E.P.; Niswander, L.; Watson, D.; Fall, R.R. *Chemosphere*. **1980**, *9*, 733-740.
96. Feng, P.C.C. *Pestic. Biochem. Physiol.* **1991**, *40*, 136-142.
97. Zablotowicz, R.M.; Hoagland, R.E.; Locke, M.A. In *Bioremediation Through Rhizosphere Technology*; Anderson, T.A.; Coats, J.R. Eds.; ACS Symposium Series 563; ACS; Wash., DC, 1994; pp184-198.
98. Zablotowicz, R.M.; Hoagland, R.E.; Locke, M.A.; Hickey, W.J. *Appl. Environ. Microbiol.* **1995**, *61*, 1054-1060.
99. Vuilleumier, S. *J. Bacteriol.* **1997**, *179*, 1431-1441.
100. Novak, P.J.; Christ, S.J.; Parkin, G.F. *Wat. Res.* **1997**, *31*, 3107-3115.
101. Stamper, D.M.; Tuovinen, O.H. *Crit. Rev. Microbiol.*, **1998**, *24*, 1-22.

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch005

Chapter 6

Bound Pesticidal Residues in Crop Plants: Chemistry, Bioavailability, and Toxicology

H. Sandermann, Jr.[1], N. Hertkorn[2], R. G. May[1,3], and B. M. Lange[1,4]

[1]Institutes of Biochemical Plant Pathology and [2]Ecological Chemistry,
GSF-National Research Center for Environment and Health,
D–85764, Oberschleißheim, Germany
[3]Present address: Laborärztliche Gemeinschaftspraxis, D–85051
Ingolstadt, Germany
[4]Present address: Institute of Biological Chemistry, Washington State
University, Pullman, WA 99164–6340

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch006

A high percentage of the radioactivity from labelled pesticides is often incorporated into bound (non-extractable) plant residues. Recent progress on plant endogeneous binding partners, linkage types as well as microbial and animal bioavailability is summarized. A mathematical model calculating the incorporation and bioavailability rates indicates that bound residue fractions can be responsible for a significant pesticidal exposure of animals and man.

Plant metabolic studies with radioactively labelled pesticides usually document a bound residue fraction that is nonextractable with common aqueous and organic solvents. Initial attempts to characterize the chemical structure and bioavailability of this fraction have been described at an early symposium of the American Chemical Society *(1)*. A number of reviews on the subject are available *(2-6)*. The amount of nonextractable pesticidal radioactivity has ranged from below 1% to more than 70%. Some of the higher incorporation rates observed in intact plants and in plant cell suspension cultures are summarized in Table I. In many cases, the parent pesticide and its soluble metabolites initially predominate in plant extracts, but over time the pesticidal bound residue fraction increases and usually remains persistent *(1-6)*. For example, the total residue of [^{14}C]-maleic hydrazide consisted of 90% parent compound and only 3% bound residue at 1.25 days post-treatment, but at 28 days

Table I. Incorporation Rates of Radiolabelled Pesticides into Nonextractable Residue Fractions of Whole Plants and Plant Cell Suspension Cultures

Whole plant studies	*Incorporation rate*[a,b] *(% of initial ^{14}C)*
propanil	38
atrazine	50
nitrofen	50
bentazon	70
2,4-D	70

Cell culture studies	*Incorporation rate*[a,c] *(% of initial ^{14}C)*
benzo[a]pyrene	15
diquat	21
pentachlorophenol	40
3,4-dichloroaniline	70

[a] Units are % of initial ^{14}C; [b] Data from reference *(2)*; [c] Data from reference *(8)*.

post-treatment, the residue contained 16% parent compound and 33% bound residue *(7)*.

A Commission of the International Union of Pure and Applied Chemistry (IUPAC) recently defined bound xenobiotic residues of plant and animal origin as follows: *"A xenobiotic bound residue is a residue which is associated with one or more classes of endogenous macromolecules. It cannot be dissociated from the natural macromolecule using exhaustive extraction or digestion without significantly changing the nature of either the exocon or the associated endogenous macromolecules" (6)*. This definition corresponds to previous definitions *(1)*. However, as will become apparent in the present article, future definitions of bound xenobiotic residues will probably ask for the demonstration of a covalent linkage in order to differentiate from xenobiotic fragments that are physically bound, e.g., by adsorption or occlusion. The IUPAC Commission has emphasized two investigations, where, in contrast to most of the previous literature *(1-4)*, high animal bioavailabilities were reported for a plant *(9)* and an animal bound residue *(10)*. This has raised the need to assess the toxicological relevance of bound pesticidal residues for humans and other animals as well as the ecotoxicological relevance for non-target organisms. Recent results are summarized here as a first basis for this type of risk assessment. Work from the authors' laboratory is emphasized, because other studies have recently been reviewed *(6)*.

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch006

Differentiation Between Xenobiotic and Natural Labelling

The IUPAC definition given above requires that the bound residue contain the parent pesticide or a xenobiotic fragment thereof. However, there are several examples in literature *(3)* where pesticides were metabolized by the plant or by microorganisms to simple compounds such as CO_2 or HCHO. The latter were then assimilated into soluble and nonextractable natural plant products, e.g. by photosynthesis. In such cases, the residue carries no toxicological relevance. A recent example is given by the fungicide emamectin benzoate *(11,12)*. One method to recognize a natural type of residue consists of the isolation of starch or cellulose. It should be determined whether these macromolecules contain radioactivity, and whether upon hydrolysis the label is present in D-glucose. This would constitute firm evidence for a non-xenobiotic incorporation pathway. The detection of defined xenobiotic split products would, however, conform to the IUPAC definition *(6)*. It is important to consider the site of radioactive labelling. For example, the labelled carboxyl-group of a pesticide could easily give rise to natural labelling of the bound residue, but the remainder of the parent molecule could lead to a xenobiotic (but non-radioactive) bound residue.

Endogeneous Binding Partners

In addition to pyrolytic and hydrolytic degradation *(1-4)*, the IUPAC Commission recommended to treat bound residue fractions with cellulase, collagenase, pepsin, papain, subtilisin, and protease K *(6)*. The various proteases are important for animal studies where protein bound residues usually predominate. In plant systems, cell wall components are the predominant binding sites. A fractionation procedure developed for plant bound residues *(13)* and some representative results are summarized in Tables II and III, respectively.

Table II. Fractionation of Plant Cell Wall Components[a]

Sequential extraction step	*Eluted fraction*
Amylase	Starch
Pronase E	Protein
EGTA	Pectin
DMSO	Lignin
Potassium hydroxide	Hemicellulose
Sulfuric acid	Cellulose

[a] Adopted from references *(6,13)*.

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch006

Table III. Distribution of Radioactivity (% of total) among Plant Cell Wall Fractions[a]

Pesticide, plant cell culture[b]	*Pectins*	*Lignins*	*Hemicellulose*	*Final residue*
Pentachlorophenol, W	7	22	36	5
4-Chloroaniline, W	48	27	5	4
PCNB, W	14	43	6	5
Isoproturon, W	6	11	66	7
Glyphosate, S	23	9	8	6
Phosphinothricin, S	6	3	17	25
Maleic hydrazide, S	4	32	14	26

[a]Adopted from *(14)*; [b]W, wheat; S, soybean.

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch006

More recently, soybean cell suspension cultures were incubated for 72 h with [carboxy-^{14}C]- or [phenyl- ^{14}C]-phenoxyacetic acid. The radioactive label was predominantly incorporated into the hemicellulose and lignin fractions and to a lower extent into the pectin fraction *(15)*. Bound radioactivity from trinitrotoluene was associated with the pectin, lignin and hemicellulose fractions of bean *(16)* and wheat *(17)* seedlings. These recent studies *(15-17)*, like all previous studies employing the Langebartels/Harms procedure, failed to chemically identify the individual fractions. The latter were tentatively designated only by the procedure used. Furthermore, the polymeric nature of the solubilized plant cell wall fractions was only in a few cases examined by gel permeation chromatography, for lignin conjugates of chloroanilines *(18)*, 2,4-D *(19)*, pentachlorophenol *(20)* and trinitrotoluene *(17)*, as well as hemicellulose metabolites of pentachlorophenol *(13)* and isoproturon *(21)*. The xenobiotics appeared to be randomly incorporated. In addition, protein conjugates have been studied by SDS polyacrylamide gel electrophoresis [e.g., *(22)*]. Non-covalently associated herbicide and metabolites migrated near the dye front.

Linkage Types

Little is known about pesticidal linkage types in bound residues. The carboxyl group is the most obvious linkage type in the case of pectin conjugates (Figure 1A). However, this idea has so far not been experimentally examined. Xenobiotics could bind by nucleophilic displacement of pectin methylester groups. The lignin conjugates of chloroanilines have been thoroughly characterized. Chloroanilines are components of urea, anilide and certain carbamate herbicides whose use has generally decreased during the past decades. The initial studies in the early 80s *(3)* already indicated by mass-spectroscopic and NMR-spectroscopic analyses that the α-position of quinonemethide-intermediates was the site of nucleophilic attack, so that benzylamine linkages were formed (Figure 1B). This conclusion has stood the test of

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch006

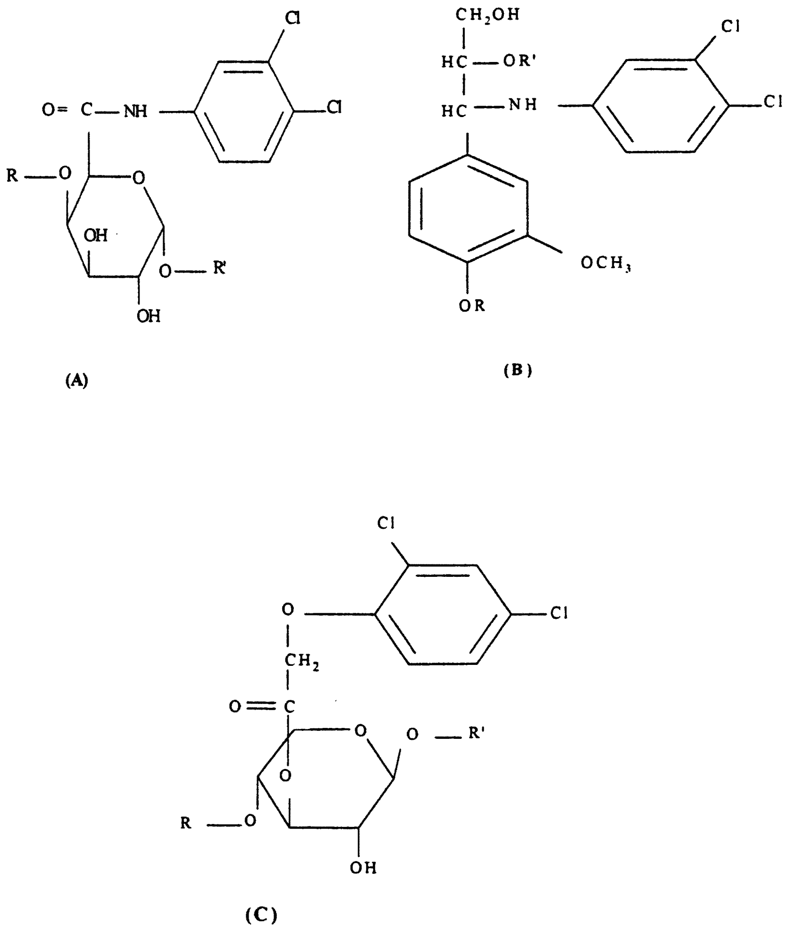


Figure 1. Proposed linkage types for (A) chloroaniline/pectin, (B) chloroaniline/lignin, and (C) phenoxyacetic acid/carbohydrate polymers. R and R' designate the attachment points of the polymer chains.

time because the most recent studies of chloroaniline/lignin conjugates employing improved polymerisation and NMR spectroscopic methods have confirmed the benzylamine linkage *(23)*. Many other nucleophilic xenobiotics, in particular the thiol-compounds generated by C-S-lyase activity from cysteinyl-conjugates may be similarly bound in α-positions of lignin. Only one report of thiol-incorporation into a defined lignin fraction seems to exist *(24)*. A hypothetical structure to explain the binding of phenoxyacetic acid *(15)* and 2,4-D *(22)* to carbohydrate polymers is shown in Figure 1C. Acylglucosides or CoA-esters may be involved as activated intermediates *(14)*.

Microbial Bioavailability

When plant bound pesticidal residues are studied in soil systems, a slow release of $^{14}CO_2$ and conversion to soil-type humic substances is observed. This has, for example, been described for plant bound residues derived from 4-chloroaniline and 2,4-dichlorophenol *(25)* as well as from isoproturon *(26)* and glyphosate *(27)*. 4-Chloroaniline and 2,4-dichlorophenol are components of herbicides such as monuron and 2,4-D, respectively. The lignin conjugates of chloroanilines were employed to answer the question whether a lignin-degrading fungus could release the chloroanilines during lignin mineralization or co-mineralize them. It was discovered in 1985, that the white-rot fungus, *Phanerochaete chrysosporium*, released $^{14}CO_2$ in high yield from lignins carrying a natural ^{14}C-label and from non-labelled lignin metabolites with bound ^{14}C-chloroanilines (28). The free chloroanilines were also mineralized with about 65% yield *(28)*. Two other reports *(29,30)* in 1985 also documented the high mineralization capacity of *P. chrysosporium* for free polycyclic and chlorinated hydrocarbons. More recently, the fungus has been shown to mineralize various native plant pesticidal bound residues, including a residue that had been shown to be non-bioavailable in animals *(31)*. The pathway of mineralization by the fungus is still under study, and white-rot fungi are also examined for bioremediation of contaminated soil and water. Special care must be exerted because the lignin peroxidase of *P. chrysosporium* can generate highly toxic azobenzenes and perhaps dioxins *(32)*. Mineralization of chloroanilines proceeds via unusual α-ketoglutaryl and succinimide conjugates of the chloroanilines so that the chloroaniline amino group is protected. This favors mineralization rather than oligomerization *(33)*. *P. chrysosporium* has been found to be inactivated when exposed to soil systems. We have therefore developed a two-stage fermentation system where contaminated soil is first eluted with the detergent Tween 80. The eluate is pumped into a second vessel containing the fungal culture that had been induced by N-limitation. Mineralization of benzo[a]-pyrene and other polycyclic hydrocarbons was low under these conditions, but oxidative activation and formation of polymers was successful in high yield. In this way a highly polluted gas-work soil from Munich was detoxified by more than 95% *(34)*.

Animal Bioavailability

Plant pesticidal bound residues in many cases have negligible animal bioavailability *(1-4)*. However, it was discovered in 1990, using chemically defined chloroaniline/lignin conjugates, that 65% - 68% bioavailability existed in rats *(9)*. It was also discovered that most of the bioavailable radioactivity was released in simple model experiments that simulated the acidic stomach conditions of animals and humans (0.1 N HCl, 37° C). In this treatment, the bioavailable residue fractions showed an immediate 'burst' release of about 30% of the bound radioactivity. This was followed by a further slow linear release of radioactivity (Figure 2). A subsequent study with a 3,4-dichloroaniline lignin metabolite fraction from wheat showed only 10% - 20%

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch006

bioavailability in rats and sheep. The simulated stomach treatment failed to produce the 'burst' release phase *(35)* (Figure 2). No release of ^{14}C from the bound residue fraction occurred in the water control. A slow release of 3% - 5% of bound radioactivity was also produced under simulated stomach conditions with the bound residue fractions of maleic hydrazide *(36)* and isoproturon *(21)*. Again, no release of ^{14}C occurred in the water controls. One possible limitation of these studies is that labile linkages of bound pesticidal moieties may have been broken in the procedures used to isolate the cell wall fractions. It has, until recently, remained an open question whether the initially observed high animal bioavailability *(9)* was relevant for field situations. A recent study employing various NMR-spectroscopic methods has partially answered this question *(23)*. Chloroaniline/lignin conjugates were prepared at various molar ratios by improved enzymatic procedures. A 'burst' release in the simulated stomach experiment was only obtained when the chloroaniline incorporation rate was higher than 20 mol%. This agreed with the high incorporation rates of the initial bioavailability studies *(9)*. When lower molar ratios of chloroanilines were used or when a 1:1 model compound of a lignin monomer and aniline was tested, no 'burst' release occurred *(23)*. ^{15}N-NMR spectroscopy showed a simple spectrum corresponding to the benzylamine binding type. At high molar ratio, there was a complex spectral pattern, but spectroscopic control experiments indicated that the benzylamine linkage still was the only linkage type present. It was concluded that special conformers of the lignin metabolites were present at high incorporation ratios, and that the 'burst' release of chloroanilines was due to anchimeric assistance *(23)*. The required molar incorporation ratios of above 20 mol% were orders of magnitude above field residues which are typically below 0.1 mol%. Therefore it is likely that the initial observation of high bioavailability *(9)* was due to a laboratory artifact. Several examples of high animal bioavailability have been described for bound residue fractions of seeds. Bound residues of deltamethrin, dieldrin, pirimiphosmethyl, malathion, diflubenzuron, and fenvalerate were up to 80% bioavailable *(5,37)*. However, the chemical nature of the seed bound residue fractions was not clarified, so these reports of high bioavailability are still incomplete.

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch006

Toxicology

As an example of regulatory rules, the U.S. Environmental Protection Agency has decided that characterization of bound pesticidal residues in plants is not required for nonextractable residues that are less than 0.05 ppm parent equivalents or 10% of the total radioactive residue. Where toxicological concerns are evident or the nonextractable residue is larger and cannot be identified, then bioavailability studies may be requested *(6)*. Incorporation rates are above 10% in many cases *(1-4)*. The pesticidal equivalents in the bound residue may under agricultural conditions (i.e. harvest several weeks after pesticide application) be orders of magnitude higher than the residue of parent pesticides that may be close to zero. For model calculations, such observations should be combined with the extent of bioavailability, at least the 3%-10% estimate of simulated stomach conditions. Pesticidal exposure in animals, man and other non-target organisms could therefore be much higher from the bound residue fraction than from residual parent pesticide.

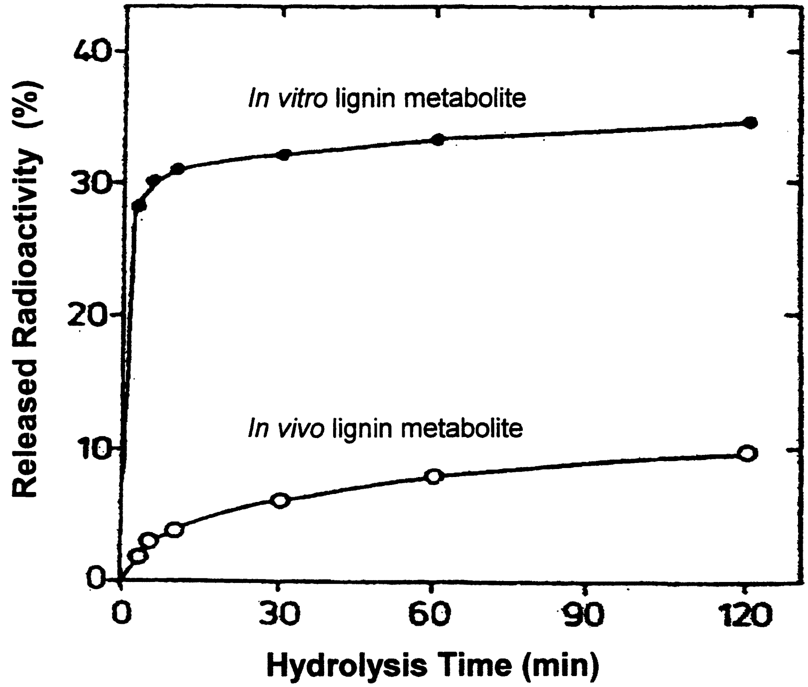


Figure 2. Mild acid hydrolysis under simulated stomach conditions (0.1 N HCl, 37° C). A synthetic in-vitro chloroaniline/lignin conjugate and a native chloroaniline/wheat lignin metabolite fraction produced in-vivo are shown in the upper and lower curve, respectively [modified from (35)].

This is in contrast to most current tolerance values for pesticide residues that are only based on parent compound. There seems to be no example where pesticidal compounds released from bound residue fractions were more toxic than the parent pesticide. Release of the parent pesticide was quite often observed. For example, the mutagenic and carcinogenic pesticide, maleic hydrazide, accounted for 80%-90% of the radioactivity released from bound soybean residues under simulated stomach conditions *(36)*. These various observations support the decision of the US-EPA to regulate bound pesticidal residues *(6)*. A toxicologically relevant pesticide exposure from bound residues appears likely in certain cases.

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch006

Conclusions

Progress since the initial ACS symposium in 1975 *(1)* has been slow. The reviewed results on linkage types, animal and microbial bioavailability and potential toxicology still allow no generalizations. Bound residues now appear to be toxicologically relevant in certain cases so that further research is necessary. This is also true concerning the long-term fate of pesticidal plant bound residues in aquatic and terrestrial environments.

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch006

References

1. Kaufman, D. D.; Still, G. G.; Paulson, G. D.; Bandal, S. K., Eds.; *Bound and Conjugated Pesticide Residues;* ACS Symposium Series 29, American Chemical Society: Washington DC, 1976.
2. Hatzios, K. K.; Penner, D.; *Metabolism of Herbicides in Higher Plants*; Issues and Concepts in Contemporary Biology; Burgess Publishing Company: Minneapolis, MN, 1982.
3. Sandermann, H.; Scheel, D.; v.d. Trenck, T. *J. Appl. Polymer Sci.* **1983**, *37*, 407-420.
4. Pillmoor, J. B.; Roberts, T.R. In *Progress in Pesticide Biochemistry and Toxicology*, Hutson, D.H.; Roberts, T.R., Eds.; J. Wiley, New York, 1985; Vol. 4, pp 86-101.
5. Kacew, S.; Akhtar, M.H.; Khan, S.U. Proc. Soc. Exper. Biol. Med. 1996, *211*, 62-68.
6. Skidmore, M. W.; Paulson, G. D.; Kuiper, H. A.; Ohlin, B.; Reynolds, S. *Pure & Appl. Chem.* **1998**, *7*, 1423-1447.
7. Frear, D. S.; Swanson, H. R. *J. Agric. Food Chem.* **1978**, *26*, 660-666.
8. Komoßa, D.; Langebartels, C.; Sandermann, H. In *Plant Contamination. Modeling and Simulation of Organic Chemical Processes.* Trapp, S.; McFarlane, J. C., Eds., Lewis Publ.: Boca Raton, 1995, pp. 69-103.
9. Sandermann, H.; Arjmand, M.; Gennity, I.; Winkler, R.; Struble, C.; Aschbacher, P. *J. Agric. Food Chem.* **1990**, *38*, 1877-1880.
10. Lu, A. Y. H.; Miwa, G. T.; Wislocki, P. G. *Rev. Biochem. Toxicol.* **1988**, *9*, 1-28.
11. Syintsakos Allen, L.; Wrzesinksi, C. L.; Feely, W. F.; Doss, G. A.; Crouch, L.S. *J. Agric. Food Chem.* 1997, *45*, 4131-4136.
12. Feely, W. F.; Crouch, L.S. *J. Agric. Food Chem.* **1997**, *45*, 2758-2762.
13. Langebartels, C.; Harms, H. Ecotox. *Environ. Safety* **1985**, *10*, 268-279.
14. Sandermann, H.; Haas, M.; Meßner, B.; Pflugmacher, S.; Schröder, P.; Wetzel, A. In *Regulation of Enzymatic Systems Detoxifying Xenobiotics in Plants;* Hatzios K. K., Ed.; Kluwer: Dordrecht, **1997**; pp 211-231.
15 Laurent, F. M. G.; Scalla, R. *Pestic. Sci.* **1999**, *55*, 3-10.
16. Sens, C.; Scheidemann, P.; Klunk, A.; Werner, D. *Environ. Sci. & Pollut. Res.* **1998**, *5*, 202-208.
17. Sens, C.; Scheidemann, P.; Werner, D. *Environ. Poll.* **1999**, *104*, 113-119.

18. Arjmand, M.; Sandermann, H. *Z. Naturforsch.* **1986**, *41*, 206-214.
19 Scheel, D.; Sandermann, H. *Planta* **1981**, *152*, 253-258.
20. Scheel, D.; Schäfer, W.; Sandermann, H. *J. Agric. Food Chem.* **1984**, *32*, 1237-1241.
21. Gläßgen, W. E.; Komoßa, D.; Bohnenkämper, O.; Haas, M.; Hertkorn, N.; May, R. G.; Szymczak, W.; Sandermann, H. *Pestic. Biochem. Physiol.* **1999**, *63*, 97-113.
22. Scheel, D.; Sandermann, H. *Planta* **1981**, *152*, 248-252.
23. Lange, B.M.; Hertkorn, N.; Sandermann, H. *Environ. Sci. & Technol.* **1998**, *32*, 2113-2118.
24. Schäfer, W.; Sandermann, H. *J. Agric. Food Chem.* **1988**, *36*, 370-377.
25. Pogány, E.; Pawlizki, K.-H.; Wallnöfer, P. R. *Chemosphere*, **1990**, *21*, 349-358.
26. Lehr, S.; Gläßgen, W. E.; Sandermann, H.; Beese, F.; Scheunert, I. *Intern. J. Environ. Anal. Chem.* **1996**, *65*, 231-243.
27. Von Wirén-Lehr, S.; Komoßa, D.; Gläßgen, W. E.; Sandermann, H.; Scheunert, I. *Pestic. Sci.* **1997**, *51*, 436-442.
28. Arjmand, M.; Sandermann, H. *J. Agric. Food Chem.* **1985**, *33*, 1055-1060.
29. Bumpus, J. A.; Tien, M.; Wright, D.; Aust, S. D. *Science* **1985**, *228*, 1434-1436.
30. Eaton, D. C. *Enzyme Microb. Technol.* **1985**, 7, 194-196.
31. May, R. G.; Sparrer, I.; Hoque, E.; Sandermann, H. *J. Agric. Food Chem.* **1997**, *45*, 1911-1915.
32. Pieper, H. D.; Winkler, R.; Sandermann, H. *Angew. Chem. Int. Ed. Engl.* **1992**, *31*, 68-70.
33. Sandermann, H.; Heller, W.; Hertkorn, N.; Hoque, E.; Pieper, D.; Winkler R. *Appl. Environ. Microbiol.* **1998**, *64*, 3305-3312.
34. May, R. G.; Schröder, P.; Sandermann, H. *Environ. Sci. Technol.* **1997**, *31*, 2626-2633.
35. Sandermann, H.; Musick, T. J.; Aschbacher, P. W. *J. Agric. Food Chem.* **1992**, *40*, 2001-2007.
36. Komoßa, D.; Sandermann, H. *J. Agric. Food Chem.* **1995**, *43*, 2713-2715.
37. Akay, M. T.; Yilmazoglus, G.; Yasacan, S.; Türk, H.; Kolanyaka, D. *Xenobiotika*, **1992**, *22*, 293-302.

Downloaded by UNIV OF GUELPH LIBRARY on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch006

Downloaded by OHIO STATE UNIV LIBRARIES on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch007

Chapter 7

A Review of Strategies to Engineer Plant Tolerance to the Pyridine Herbicides

Paul C. C. Feng and Thomas G. Ruff

Agricultural Research, Monsanto Company, 700 Chesterfield Village Parkway, St. Louis, MO 63198

Metabolic deactivation was used to engineer plant tolerance to thiazopyr and other pyridine herbicides. De-esterification was selected as the pathway, after confirmation that the resulting acid metabolite lacked herbicidal activity. We examined de-esterification via mixed function oxygenases, but selected esterase based on its many desirable features. Esterase-mediated hydrolysis is a single-step deactivation with no requirement for cofactors. Esterases hydrolyzed not only thiazopyr but also other pyridines and demonstrated sufficient catalytic activity to deactivate the level of thiazopyr residues encountered in plants. By tracking the pyridine-esterase activity, a novel 60 kDa esterase (RLE3) was purified from rabbit liver, and a cDNA was cloned based on amino acid sequence. The esterase cDNA, when expressed in insect cells, demonstrated activity against the pyridines. Stable plant transformation was conducted in tomato and tobacco with kanamycin selection. Analysis of leaf tissues from R_0 plants confirmed the expression of the 60 kDa protein and *in vitro* pyridine-esterase activity. Transgenic seedlings expressing the RLE3 esterase showed *in vivo* hydrolysis of the pyridines to the acid. In growth chamber and greenhouse tests, seeds from transgenic tomato and tobacco showed enhanced tolerance to thiazopyr over the control during germination. Thiazopyr tolerance was directly correlated to pyridine-esterase expression.

Introduction

Pyridines are a family of herbicides useful for controlling narrow-leaf and small seeded broad-leaf weeds under pre-emergence application. Thiazopyr, MON 2300 and MON 14300 (Figure 1) all share a similar pyridine backbone with a methylester group at the

In Pesticide Biotransformation in Plants and Microorganisms; Hall, J., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2000.

pyridyl-3 position and a distinguishing functional group at the pyridyl-5 position. Dithiopyr has a similar pyridine backbone with methylthioester groups at both pyridyl-3 and -5 positions. Cell biology studies using dithiopyr indicate that disruption of cell division is the mode of action of the pyridine herbicides (*1*). Seeds germinated in the presence of pyridine herbicides show characteristic inhibition of root elongation and swelling of meristematic zones. Pyridine herbicides are reported to disrupt microtubule organization during cell division (*2*).

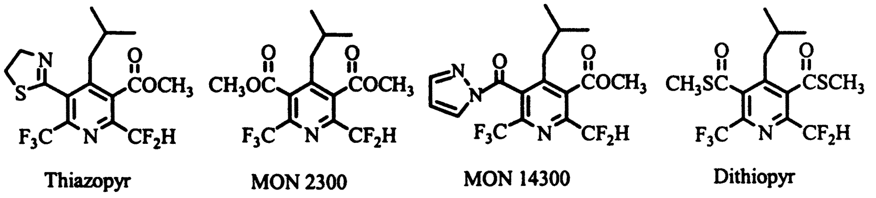


Figure 1. Structures of representative pyridine herbicides

Metabolism studies of thiazopyr in both animals (*3*) and plants (*4*) have demonstrated rapid oxidations at the sulfur or carbon atoms in the thiazoline ring. Oxidation reactions produced many metabolites, some of which remained herbicidally active. In contrast, the acid metabolite, resulting from de-esterification, was virtually void of herbicidal activity (*5*). Using extracts from animal livers as model systems, we demonstrated that de-esterification can occur through either oxidation (*6*) or hydrolysis (*5*). In comparison, plants appeared to de-esterify thiazopyr exclusively via the oxidation pathway, and we demonstrated enhancement of thiazopyr activity by use of mono-oxygenase inhibitors (*7*).

In this manuscript, we describe our strategies to engineer plant tolerance to thiazopyr and the pyridines. We selected esterase-mediated hydrolysis as the pathway for deactivation, and proceeded to purify, clone and express a novel pyridine-esterase in plants (*8*). The proof of concept was realized with demonstration of whole plant tolerance to thiazopyr.

Materials and Methods

The studies employed ^{14}C-(pyridine-4)-labeled thiazopyr, MON 2300, MON 14300 or dithiopyr which were custom synthesized by the radiosynthesis group in Monsanto Co (St. Louis, MO) with greater than 98% purity as determined by HPLC analyses. Halosulfuron, ^{14}C-labeled in the pyrazole ring, was also provided by Monsanto Co. Esterase substrates (*p*-nitrophenyl butyrate, 4-methyl umbelliferyl butyrate and 5-bromo-4-chloro-3-indolyl acetate) as well as liver esterases (carboxyl esterase; carboxylic-ester hydrolase; EC 3.1.1.1) from porcine and rabbit were obtained from Sigma Chem. Co (St. Louis, MO).

Downloaded by OHIO STATE UNIV LIBRARIES on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch007

In Vitro Oxidation by Rat Liver Microsomes

Liver enzyme preparations were obtained from male Sprague-Dawley and Long Evans rats following a previously reported procedure (*6*). Rat liver microsomes, previously stored at -80 °C, were freshly thawed for each incubation. In each 0.5 mL reaction volume, liver microsomes (1.0 - 5.0 mg protein) were incubated with the ^{14}C-pyridines (0.015 mM) in 0.1 M phosphate buffer, pH 7.4, containing $MgCl_2$ (5 mM) and an NADPH-generating system (NADP, 2.5 mM; glucose-6-phosphate, 10 mM; glucose-6-phosphate dehydrogenase, 3 units). Reactions were conducted at 37 °C and were quenched with an equal volume of 1% trifluoroacetic acid in methanol. Following centrifugation, supernatant fraction was analyzed using an HPLC equipped with a radioactivity detector (RAD) (*6*). Control incubations were conducted in the absence of the NADPH-generating system.

In Vitro and *In Vivo* Assays for Esterase Activity

Esterase activity was measured *in vitro* by conversion of each ^{14}C-pyridine to its corresponding acid (*5, 8*). Pyridine-esterase activity was assayed during protein purification and from tissue extracts of transgenic plants. The homogenizing buffer for plant tissues consisted of 100 mM Tris pH 8.0, 1 mM EDTA, 1 mM DTT, and 10% glycerol in water. Plant tissues (1 g/mL buffer) were homogenized in conical centrifuge tubes using a spinning pestle at 4 °C. Following centrifugation (12,000 x g), the supernatant (250 μL) was assayed immediately for esterase activity by incubation with thiazopyr or MON 14300 (0.03 mM) in 0.1 M Tris pH 8.0 buffer (50 μL) at 37 °C. The reaction (100 μL) was stopped by addition of 1% trifluoroacetic acid in acetonitrile (v/v, 150 μL) to generate a time course (0.5 to100 h) for formation of the acid metabolite. Following centrifugation (12,000 x g for 3 min), the supernatant was analyzed by HPLC/RAD to resolve the acid from the parent herbicide and other metabolites. Control incubations in the absence of the enzyme recovered thiazopyr or MON 14300. Esterase activity was also assayed during protein purification, except a single time-point (1-2 h) of incubation was selected within the linear range of reaction. *In vitro* assays conducted with colorimetric (*p*-nitrophenyl butyrate) or fluorescent substrates (4-methyl umbelliferyl butyrate and 5-bromo-4-chloro-3-indolyl acetate) were used as quick qualitative assays for the detection of general esterase activity.

Esterase activity was also monitored *in vivo*. ^{14}C-Thiazopyr or MON 14300 (0.003-0.01 μmol in acetonitrile) was applied directly to shoots and cotyledons of young seedlings. After 2 days in a growth chamber, tobacco or tomato seedlings were washed with methanol and water, and homogenized (Tissuemizer by Tekmar) in 5% (v/v) trifluoroacetic acid in acetonitrile (fresh w/v ratio of 2/1). Homogenates were clarified by centrifugation (12,000 x g) and supernatants analyzed by HPLC/RAD for the formation of the acid metabolite. Three individual HPLC methods were employed for analysis of the corresponding acid metabolites from thiazopyr, MON 14300 or halosulfuron in plant tissue extracts (*8*).

Purification of Pyridine-esterases from Porcine and Rabbit Liver

Porcine and rabbit liver esterases, obtained commercially as $(NH_4)_2SO_4$ suspensions, were desalted using a disposable Sephadex G25 size exclusion column (Pharmacia Biotech). Proteins (1 to 10 mg) were initially separated on a strong anion exchange column

Downloaded by OHIO STATE UNIV LIBRARIES on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch007

(Mono Q, Pharmacia Biotech) using a KCl gradient (*8*). Effluent fractions were assayed for protein using Coomassie dye (Bio-Rad Laboratory) and for pyridine-esterase activity. Protein purity was determined by SDS-PAGE on 10 to 15% gradient gels (Pharmacia Biotech) with visualization of proteins by silver staining. Following anion exchange chromatography, fractions with the highest purity and activity were pooled. After desalting, the pooled fractions were further separated by isoelectric focusing (IEF) chromatography on a Mono P column (Pharmacia Biotech). IEF chromatography was conducted at different pH ranges (3 to 9, 4 to 7, 5.5 to 6.5 or 4.5 to 5.5) using appropriate combinations of Polybuffers. Effluent fractions from IEF chromatography were analyzed for protein and pyridine-esterase activity. Protein purity of the fractions was determined by SDS-PAGE and IEF-PAGE.

Downloaded by OHIO STATE UNIV LIBRARIES on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch007

PCR Cloning of Rabbit Liver Esterase cDNAs

Based on the published amino acid sequence of rabbit liver esterase isozyme 1 (RLE1) (*9*), we designed 64-fold degenerate primers at the amino and carboxy termini of the mature protein utilizing nucleotide degeneracy only in the 3' region of the primer. The amino terminal primer contained an *Nco I* restriction site encoding an initiating methionine residue for expression. The carboxy terminal contained a termination codon and the restriction sites *Xba I* and *Sac I* for cloning. The primer for the amino terminal was 5'GCACCATGGCCCACCCCTCCGCACCACCTGTGGTTGACACTGTNAARGGNAGT and the primer for the carboxy terminal was 5'CGCTCTAGAGCTCTACAGYTCGATRTGYTCNGTYTC.

Rabbit liver poly A+ RNA (2 μg) was reverse transcribed with AMV reverse transcriptase to generate first strand cDNA for use as template in the PCR reaction. An equivalent of 80 ng of starting material was used for 30 cycles of PCR with Taq polymerase in segments of 94 °C (1 min), 25 °C (2 min) and 72 °C (2 min). Products in the 1300 to 1900 base pair range were recovered and reamplified as described above with a more stringent annealing temperature of 35 °C. The reaction yielded a single band of the predicted molecular weight. This esterase clone was completely sequenced in both directions, utilizing the Sanger method and the Sequenase reagents (US Biochemical). The deduced amino acid sequence from the cloned esterase showed divergence from the published RLE1 and therefore was designated as isozyme 3 (RLE3).

A synthetic endoplasmic reticulum (ER) signal sequence was designed based on a published cDNA sequence of a homologous esterase (*10*). Oligonucleotides of 56 and 57 nucleotides were synthesized to encode both strands of the signal sequence. An alanine residue was added downstream of the initiating methionine residue in order to incorporate the *Nco I* restriction site.

Using the cloned *rle3* cDNA as the template, we carried out two independent PCR events to generate a cDNA for *rle1*. Two oligonucleotides were designed such that the 3'-end was homologous to RLE3 sequences flanking the 24 amino acid-divergent region, and the 5' end encoded for the divergent region of *rle1*. The two primers were designed with opposite orientations and contained an overlapping segment in the *rle1* divergent region with a unique *Cla I* restriction site. Two PCR reactions were run independently utilizing *rle3* as the template producing two double stranded DNAs encoding for two halves of *rle1*. The double-stranded DNAs were recovered into pBluescript, trimmed with *Cla I* digestion, and assembled through blunt-end ligation to produce the *rle1* (*8*).

Expression of *rle1* and *rle3* cDNAs in Insect Cells and in Plants

Rabbit liver esterase cDNAs (*rle1* and *rle3*) with an ER signal sequence were expressed in insect cells using the baculovirus system under the control of the polyhedrin promoter. Transfer vector DNA (2 µg) along with 1 µg of baculovirus genomic DNA were transfected into *Spodoptera frugiperda* clone 9 (SF9) cells by the standard calcium phosphate method. After 5 days of transfection at 27°C, cells were precipitated by centrifugation at 3000 x g. The cell free supernatant and the cell lysate were tested for esterase activity against thiazopyr or MON 14300, and for the 60 kDa esterase protein by Western blot analysis (*5*). The recombinant baculoviruses were confirmed by dot blot DNA hybridization and purified by three rounds of plaque purification.

The vector for stable expression in tomato (UC82B) and tobacco (Samson) contained the FMV (figwort mosaic virus) promotor driving ER-*rle3*. The vector also contained the NPTII (neomycin phosphotransferase) gene as the selectable marker. Vector was introduced into plants using the well-established *Agrobacterium* transformation (*11*). Callus tissues on agar were selected based on kanamycin tolerance. Shoots from the calli were isolated for root induction and subsequently transfered to soil.

Downloaded by OHIO STATE UNIV LIBRARIES on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch007

Assays for Thiazopyr Tolerance

Transgenic tobacco R_1 seeds (line 37432) were sterilized with 0.25% sodium hypochlorite solution and repeatedly washed with water. Just prior to solidification of agar (~45 °C), a stock solution of thiazopyr in DMSO (1% final concentration) was added; the final concentrations of thiazopyr were 0, 0.05, 0.1, and 0.5 µM. The liquid agar was vortexed and dispensed (3 mL) into glass test tubes. Seeds (~ 12 per test tube) were placed on the surface of solidified agar and stored in a growth chamber (25 °C, 12 h day/night cycle). Root development and length were monitored visually. Untransformed tobacco seeds from the Samson variety were used as control plants.

Tomato seeds (R_1 of 8255 and 8256, and control UC82B) were planted in 100 cm^2 pots (12 seeds per pot) in artificial Metromix soil at a depth of 0.5 cm. Pots were watered and oversprayed with a 50% acetone/water solution (2 mL) containing thiazopyr at use rates of 0, 34, 67, 140, or 280 g ai/ha. Pots were placed in a growth chamber (12 h photoperiod; 60% humidity, 25 °C night/29 °C day; 600 $\mu E/s/m^2$ light) with daily watering by subirrigation.

Similar studies were also conducted in the greenhouse with pre-plant incorporation of thiazopyr into the soil. Tomato seeds were planted in 100 cm^2 pots filled with sterilized silty loam soil (510 g). Seeds were placed on top of soil, and covered with additional soil (135 g) that was premixed with thiazopyr at use rates equivalent to of 0, 34, 45, 67, or 140 g ai/ha. Greenhouse growing conditions were: 29 °C day to 21 °C night with supplemental lighting as needed to maintain 500 $\mu E/s/m^2$.

Results

Our major objective was to demonstrate that plant tolerance to thiazopyr can be achieved via a metabolic deactivation mechanism. Although our focus was on thiazopyr, we were very much interested in identifying a mechanism that would be applicable to other pyridines. We considered various parameters that would constitute an ideal pathway of metabolic deactivation. The pathway should be a single detoxification step away from the parent so that no more than one enzyme would have to be introduced into plants. The enzyme should be stable and abundant to facilitate purification and cloning. Above all, the enzyme should have favorable kinetic parameters (Km and Vmax) so as to rapidly degrade thiazopyr residues encountered in plants under commercial rates of application. Finally, the deactivated metabolite should preferably be a terminal metabolite so as to eliminate further complication from metabolic re-activation. Although there are many other desirable features for a deactivation enzyme, we considered these to be most important.

Downloaded by OHIO STATE UNIV LIBRARIES on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch007

Confirmation of Thiazopyr as the Target of Deactivation

Pre-emergence application of ^{14}C-thiazopyr (0.1-0.5 kg ai/ha) to soil produced characteristic swelling of roots and shoots, and severe stunting in soybean and corn seedlings. Thiazopyr was slowly degraded in soil, accounting for 93% of the residue 2 weeks after treatment. Combustion analysis showed very low levels of residues in seedlings with the highest level (0.22 μg/g fresh wt) being detected in cotyledons. Although 4 to 6 metabolites were detected in seedlings, thiazopyr was identified as the major residue during germination. The predominance and the slow degradation of thiazopyr both in soil and in plants during germination confirmed thiazopyr as the primary target for deactivation.

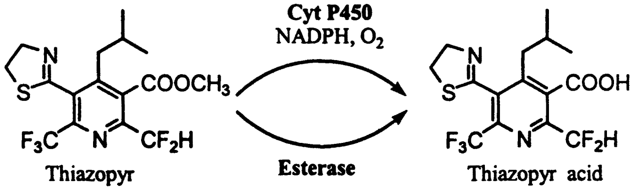


Figure 2. Potential pathways of pyridine de-esterification via oxidation or hydrolysis.

Identification of a Deactivation Pathway

The strategy was first to identify the deactivated metabolites of thiazopyr and the enzymes that were capable of such catalysis. The selected enzyme was then purified and it's peptide sequence used for the purpose of cloning the cDNA. The final steps involved the expression of the gene and demonstration of whole plant tolerance. We were initially attracted to the thiazopyr acid for its lack of herbicidal activity in plant spray tests. A critical set of experiments was conducted with thiazopyr acid to differentiate between the lack of inherent herbicidal activity versus the lack of plant uptake. We employed ^{14}C-thaizopyr acid and soybean tissue culture cells to demonstrate that the acid was readily absorbed and was about 1,000 times less active than thiazopyr (*5*).

There are two known families of enzymes capable of catalyzing de-esterification (Figure 2). The cytochrome P-450 mixed function oxygenases in the presence of NADPH and oxygen can catalyze an O-demethylation reaction producing the acid and formaldehyde (*12, 13*). De-esterification can also result from esterase-mediated hydrolysis to produce the acid and methanol (*14*). Using NADPH-fortified rat liver microsomes as a model, we examined the potential of pyridine herbicides to undergo oxidation reactions. Time course studies (Figure 3) demonstrated very rapid disappearance of thiazopyr and dithiopyr, whereas MON 14300 and MON 2300 were oxidized very slowly. Further examination revealed that the sulfur atom was the focus of oxidation in both thiazopyr (*5*) and dithiopyr (*15*). We suspect the lack of sulfur in MON 14300 and MON 2300 may have contributed to their slow rates of oxidation. Although thiazopyr was rapidly oxidized, the acid was only a minor product and many oxidized metabolites still retained herbicidal activity. The rapid oxidation of dithiopyr produced mono- and di-acid metabolites (*15*). Based on the slow oxidation of MON 14300 and MON 2300, the minor pathway of thiazopyr acid formation and the requirement for enzyme cofactors (i.e. NADPH), we concluded that de-esterification via mixed function oxygenase would not satisfy our criteria.

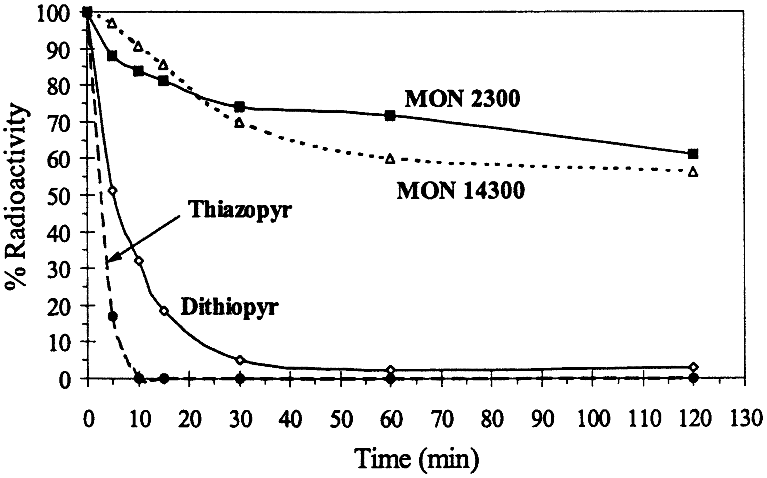


Figure 3. Time course of oxidation of pyridine herbicides by NADPH-fortified rat liver microsomes.

Time course of hydrolysis of the pyridines by porcine liver esterase is shown in Figure 4. MON 14300 and MON 2300 were rapidly hydrolyzed while thiazopyr underwent modest hydrolysis and dithiopyr little to no hydrolysis. The acid was the only metabolite from the hydrolysis of MON 14300, MON 2300, and thiazopyr. Because dithiopyr was not hydrolyzed, oxygenases will likely be required for engineering tolerance. The difference in the rates of pyridine hydrolysis (Figure 4) also suggested that greater plant tolerance should result from a rapidly hydrolyzed pyridine (i.e. MON 14300). A survey of extracts from

Downloaded by OHIO STATE UNIV LIBRARIES on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch007

animal-liver acetone powder identified many sources of thiazopyr-esterase activity (*5*). The rate of hydrolysis for thiazopyr was animal species-dependent and ranged from the highest activity in bovine to no activity in mouse. Because of commercial availability of partially purified enzymes, we selected rabbit and porcine liver esterases for further purification and analysis. We purified a pyridine-esterase from both rabbit and porcine, and chose the rabbit esterase because of its superior kinetic parameters.

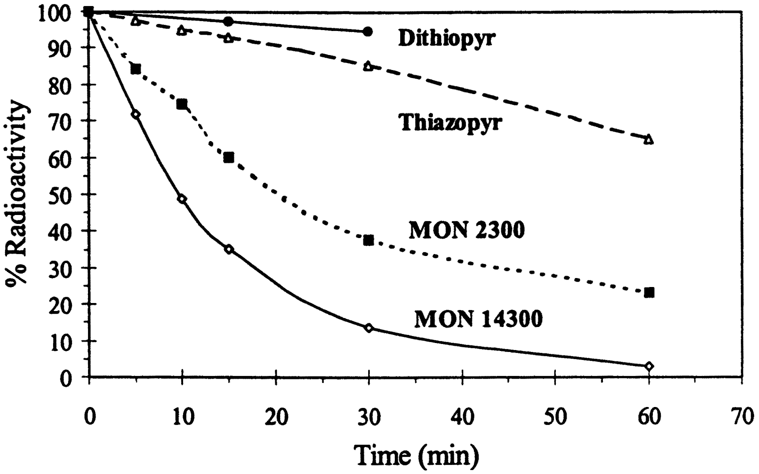


Figure 4. Time course of hydrolysis of pyridine herbicides by porcine liver esterases.

Purification of a Rabbit Liver Pyridine-esterase

A commercially available mixture of rabbit liver esterase was active against both MON 14300 and thiazopyr. MON 14300 was hydrolyzed faster than thiazopyr and underwent little to no metabolism in plants (unpublished results) which facilitated chromatographic separation of the acid metabolite. Because of these advantages, we employed MON 14300 as the substrate of choice for assaying pyridine-esterase activity. We also monitored esterase activity against thiazopyr because of its advanced stage of commercial development.

By tracking hydrolytic activity of MON 14300 and thiazopyr, a pyridine-esterase from rabbit was purified after two successive rounds of anion exchange chromatography. Analysis by SDS-PAGE demonstrated a single protein of 60 kDa molecular weight. In order to separate the individual esterase isozymes, the anion exchange fraction was further purified using isoelectric focusing (IEF) chromatography between the pH of 5.5 to 6.5. Analysis of the major IEF column fraction by IEF-PAGE (pH 4-6.5) showed two major isozymes with a pI of 6.0. Peptide sequencing yielded one N-terminal sequence for 20 amino acids with high recovery of proteins. Kinetic constants for the purified rabbit esterase against thiazopyr demonstrated a Vmax of 6.63 nmol/min/mg and a Km of 0.019 mM as calculated from the double reciprocal plot ($R^2 = 0.996$).

Downloaded by OHIO STATE UNIV LIBRARIES on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch007

The N-terminal amino acid sequences of rabbit pyridine-esterases was highly homologous to the published peptide sequence of esterase isozyme 1 of rabbit liver (RLE1) (*16*) with identity in 17 of the 18 identified residues (Table 1). In addition, RLE1 and pyridine-esterase shared an identical pI (6.0) and molecular mass (60 kDa). A second published isozyme (RLE2) of rabbit liver esterase (*17*) showed less homology to the purified pyridine-esterase than did RLE1. Preparations of RLE1 and RLE2 were obtained from J. Ozols (Univ. of Connecticut); thiazopyr hydrolysis was demonstrated with RLE1 but not with RLE2. Based on these characterizations, we deduced that the purified pyridine-esterase was in fact RLE1.

Downloaded by OHIO STATE UNIV LIBRARIES on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch007

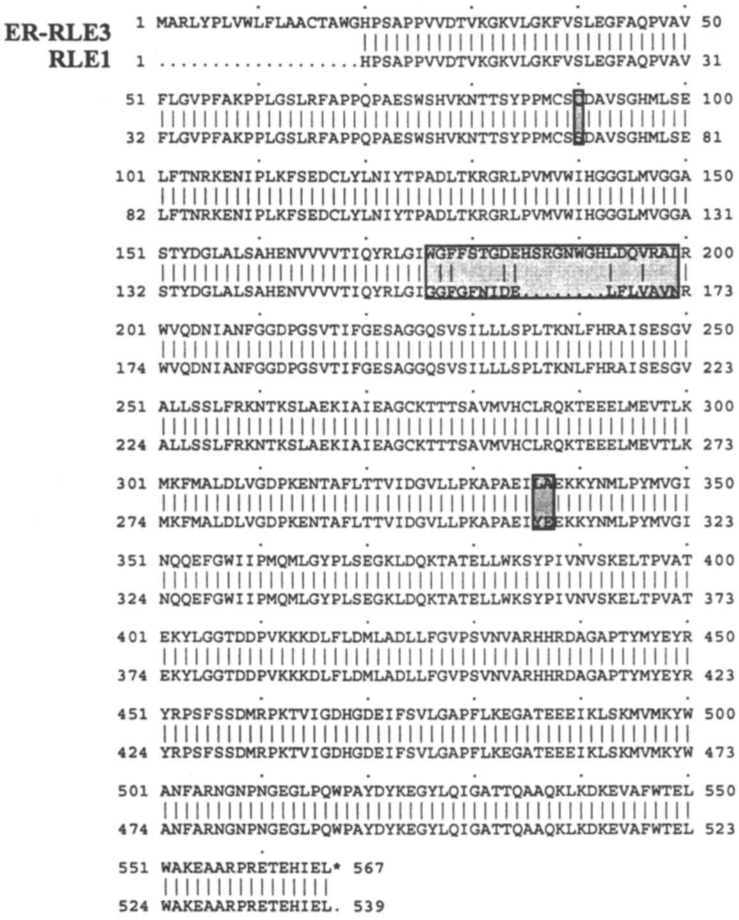
Table 1. Comparison of N-terminal amino acid residues of purified pyridine esterase (RLE3) and published esterase isozymes (RLE1 and RLE2) from rabbit liver

N-terminal amino acid	*Purified* RLE3	*Published* RLE1 (*16*)	*Published* RLE2 (*17*)
1	x	his	gln
2	pro	pro	asp
3	x	ser	ser
4	ala	ala	ala
5	pro	pro	ser
6	pro	pro	pro
7	val	val	ile
8	val	val	arg
9	asp	asp	asn
10	thr	thr	thr
11	val	val	his
12	his	lys	thr
13	gly	gly	gly
14	lys	lys	gln
15	val	val	val
16	leu	leu	arg
17	gly	gly	gly
18	lys	lys	ser
19	phe	phe	leu
20	val	val	val

NOTE: x represents unassigned residue.

PCR Cloning of Rabbit Liver Esterases (RLE1 and RLE3)

Degenerate primers for the amino and carboxy termini were designed based on the published peptide sequence of RLE1 (*16*). Reverse transcription-PCR was performed on rabbit liver mRNA with an initial round of PCR under very low stringency followed by a second round with higher stringency. This resulted in the production of a single band of the predicted size which was subcloned for further analysis. Sequence analysis of the recovered cDNA indicated that it, in fact, encoded an esterase which was different from RLE1. The derived amino acid sequence of the clone showed 97% identity to RLE1 with most of the divergence lying in one region of 24 amino acids near the amino terminus (Figure 5). Other than this divergent region, only three amino acid residues in the clone differed from RLE1.



```
                     .         .         .         .         .
ER-RLE3   1 MARLYPLVWLFLAACTAWGHPSAPPVVDTVKGKVLGKFVSLEGFAQPVAV 50
                               |||||||||||||||||||||||||||||||
   RLE1   1 ...................HPSAPPVVDTVKGKVLGKFVSLEGFAQPVAV 31
                     .         .         .         .         .
         51 FLGVPFAKPPLGSLRFAPPQPAESWSHVKNTTSYPPMCSQDAVSGHMLSE 100
            ||||||||||||||||||||||||||||||||||||||| ||||||||||
         32 FLGVPFAKPPLGSLRFAPPQPAESWSHVKNTTSYPPMCSSDAVSGHMLSE 81
                     .         .         .         .         .
        101 LFTNRKENIPLKFSEDCLYLNIYTPADLTKRGRLPVMVWIHGGGLMVGGA 150
            ||||||||||||||||||||||||||||||||||||||||||||||||||
         82 LFTNRKENIPLKFSEDCLYLNIYTPADLTKRGRLPVMVWIHGGGLMVGGA 131
                     .         .         .         .         .
        151 STYDGLALSAHENVVVVTIQYRLGIWGFFSTGDEHSRGNWGHLDQVRAIR 200
            ||||||||||||||||||||||||| ||    ||        |  |   |
        132 STYDGLALSAHENVVVVTIQYRLGIGGFGFNIDE........LFLVAVNR 173
                     .         .         .         .         .
        201 WVQDNIANFGGDPGSVTIFGESAGGQSVSILLLSPLTKNLFHRAISESGV 250
            ||||||||||||||||||||||||||||||||||||||||||||||||||
        174 WVQDNIANFGGDPGSVTIFGESAGGQSVSILLLSPLTKNLFHRAISESGV 223
                     .         .         .         .         .
        251 ALLSSLFRKNTKSLAEKIAIEAGCKTTTSAVMVHCLRQKTEEELMEVTLK 300
            ||||||||||||||||||||||||||||||||||||||||||||||||||
        224 ALLSSLFRKNTKSLAEKIAIEAGCKTTTSAVMVHCLRQKTEEELMEVTLK 273
                     .         .         .         .         .
        301 MKFMALDLVGDPKENTAFLTTVIDGVLLPKAPAEILAEKKYNMLPYMVGI 350
            |||||||||||||||||||||||||||||||||||  |||||||||||||
        274 MKFMALDLVGDPKENTAFLTTVIDGVLLPKAPAEIVPEKKYNMLPYMVGI 323
                     .         .         .         .         .
        351 NQQEFGWIIPMQMLGYPLSEGKLDQKTATELLWKSYPIVNVSKELTPVAT 400
            ||||||||||||||||||||||||||||||||||||||||||||||||||
        324 NQQEFGWIIPMQMLGYPLSEGKLDQKTATELLWKSYPIVNVSKELTPVAT 373
                     .         .         .         .         .
        401 EKYLGGTDDPVKKKDLFLDMLADLLFGVPSVNVARHHRDAGAPTYMYEYR 450
            ||||||||||||||||||||||||||||||||||||||||||||||||||
        374 EKYLGGTDDPVKKKDLFLDMLADLLFGVPSVNVARHHRDAGAPTYMYEYR 423
                     .         .         .         .         .
        451 YRPSFSSDMRPKTVIGDHGDEIFSVLGAPFLKEGATEEEIKLSKMVMKYW 500
            ||||||||||||||||||||||||||||||||||||||||||||||||||
        424 YRPSFSSDMRPKTVIGDHGDEIFSVLGAPFLKEGATEEEIKLSKMVMKYW 473
                     .         .         .         .         .
        501 ANFARNGNPNGEGLPQWPAYDYKEGYLQIGATTQAAQKLKDKEVAFWTEL 550
            ||||||||||||||||||||||||||||||||||||||||||||||||||
        474 ANFARNGNPNGEGLPQWPAYDYKEGYLQIGATTQAAQKLKDKEVAFWTEL 523
                     .
        551 WAKEAARPRETEHIEL* 567
            ||||||||||||||||
        524 WAKEAARPRETEHIEL. 539
```

Figure 5. Amino acid alignment of cloned ER-RLE3 with the published RLE1. Divergent regions are highlighted in gray.

Other subclones isolated from the PCR amplification were sequenced through this divergent region and were all identical to the original clone. Our isolated clone encoded a

Downloaded by OHIO STATE UNIV LIBRARIES on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch007

novel esterase which was designated as rabbit liver esterase isozyme 3 (RLE3). It was apparent that by using primers designed against RLE1, the PCR reactions cloned a cDNA for *rle3*. Comparison of the sequence of RLE3 with other published sequences revealed homology to a clone isolated from rat (*10*).

Although the peptide sequence of RLE3 was 97% identical to RLE1, we were concerned that the minor dissimilarity could affect enzymatic activity against thiazopyr. Since a purified preparation of RLE1 esterase did exhibit thiazopyr hydrolysis, we proceeded to construct a *rle1* cDNA. The primary difference between RLE3 and the published RLE1 sequence was in one region of 24 amino acids. Using *rle3* cDNA as the template, we carried out two independent PCR events using primers that encoded for sequences flanking the divergent region of *rle3* and the divergent region of *rle1*. Two double stranded DNA encoding for each halve of *rle1* were produced and ligated. Conversion of this cDNA to *rle1* was completed by site specific mutagenesis at three other positions where a single amino acid difference existed between RLE1 and RLE3. The entire sequence of the *rle1* cDNA was confirmed by double stranded nucleic acid sequencing.

Downloaded by OHIO STATE UNIV LIBRARIES on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch007

Insect Expression of *rle1* and *rle3* cDNAs

Of the two putative esterase cDNAs, *rle1* was designed based on the published amino acid sequence of RLE1 (*16*) and *rle3* was cloned based on primers designed from RLE1. The demonstration of pyridine-esterase activity from the protein products of these two cDNAs was accomplished by expression in insect cells. The expression of the pyridine-esterase was measured by Western blot analysis, and the enzymatic activity measured against standard colorimetric substrates, thiazopyr and MON 14300.

Mature rabbit liver esterases are glycoproteins which reside in the lumen of the endoplasmic reticulum (ER) (*16*). The cloning strategy that recovered *rle3* cDNA was based on the peptide sequence of the RLE1 mature protein which did not contain a signal sequence for ER targeting. Assuming that glycosylation and/or disulfide bond formation in the ER is critical to esterase activity, we designed an ER signal sequence based on the published oligonucleotide sequence of the rat liver esterase (*10*). We employed the standard baculovirus-mediated insect expression utilizing a transfer vector (pVL1893) with polyhedrin promoter. Western blot analysis detected high levels of the 60 kDa esterase protein in the media of both *rle1* and *rle3* cultures. The media from *rle3* culture had esterase activity against standard substrates, thiazopyr and MON 14300. This esterase activity was inactivated by boiling and also by bis(*p*-nitrophenyl)phosphate, a previously demonstrated esterase inhibitor. In contrast, the media from *rle1* culture was inactive against all esterase substrates. These results indicated that although both *rle1* and *rle3* were equally expressed in insect cells, only the RLE3 protein demonstrated esterase activity. The failure of the RLE1 protein to hydrolyze even standard esterase substrates suggests that the enzyme is not functional. The inactivity of the RLE1 esterase could be due to lack of proper folding, although RLE3 esterase which is 97% identical to RLE1 was folded correctly. The absence of hydrolysis activity in the protein product of our *rle1* cDNA and the presence of activity in the purified RLE1 esterase from J. Ozols indicate the likelihood that errors exist in the published peptide sequence of RLE1 (*9*).

Downloaded by OHIO STATE UNIV LIBRARIES on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch007

Plant Transformation of *rle3* cDNA

Stable transformation of ER-*rle3* was conducted in tomato and tobacco using the FMV constitutive promotor. The vector, which also contained the NPTII (neomycin phosphotransferase) gene as the selectable marker, was introduced using *Agrobacterium* transformation (*11*). Primary callus tissues were selected for kanamycin tolerance, and shoots were rooted and regenerated into R_0 plants. Leaf tissue from R_0 tomato and tobacco plants were assayed by Western blot (Figure 6). Expression of the 60 kDa RLE3 esterase was detected in most of the transgenic tobacco lines at levels ranging from 0.004 to 0.5% of total protein. Among 35 lines of tomato, 20 lines expressed the esterase at levels ranging from 0.002 to 0.05% of protein. Expression of RLE3 esterase was consistently higher in tobacco than in tomato plants.

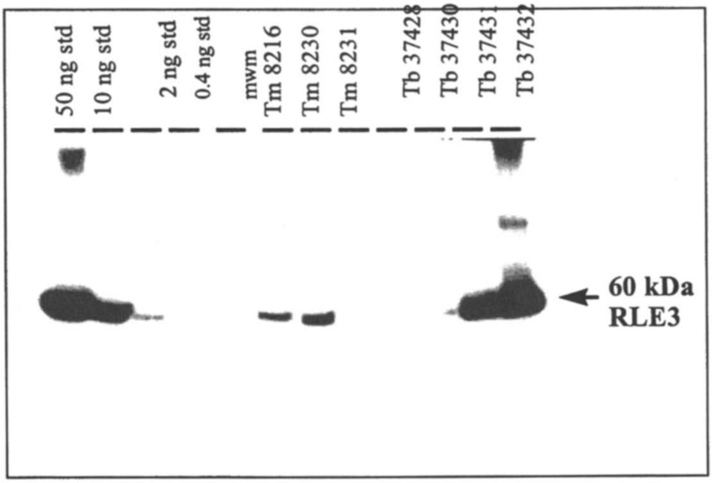


Figure 6. Detection of RLE3 esterase in tissue extracts from 3 tomato and 4 tobacco lines by Western blot analysis (Reproduced from reference 8).

In addition to leaves, RLE3 esterase was also detected in tissue extracts from seeds, roots, flowers and fruits, which is consistent with the expression pattern of the constitutive FMV promotor. Since thiazopyr is a pre-emergence herbicide acting primarily during early germination, we were particularly interested in expression of RLE3 in young seedlings. Starting with seeds from a high-expressing tobacco line (37432), we observed consistent expression of RLE3 in seeds and through 17 day-old seedlings. Ubiquitous expression of RLE3 in all plant tissues caused no visible abnormal phenotype. Transgenic tobacco and tomato plants were normal in all aspects of development from vegetative growth, flowering, fruit setting and maturation, to seed production and germination. The R_0 lines were carried through fruit maturation to obtain R_1 seeds for tolerance assays.

Detection of Plant RLE3 Esterase Activity *In Vitro* and *In Vivo*

A crucial step in our overall concept was the demonstration of enzymatic activity in the plant-expressed RLE3. Using tissue extracts from R_0 tomato and tobacco plants, we observed *in vitro* hydrolysis of MON 14300 or thiazopyr only in RLE3-expressing plants. Seedling extract from a high-expressing tobacco line was used to examine the substrate specificity against MON 14300, thiazopyr and halosulfuron (a herbicide of the sulfonylurea family containing a methylester functional group). Results indicated that RLE3 esterase clearly hydrolyzed both MON 14300 and thiazopyr, but not halosulfuron (*8*). As observed in the crude rabbit esterase preparation, MON 14300 was hydrolyzed at a faster rate than thiazopyr. These results suggest that although animal liver esterases are generally regarded as having a broad substrate specificity, the RLE3 esterase appeared to possess some specificity towards the pyridines.

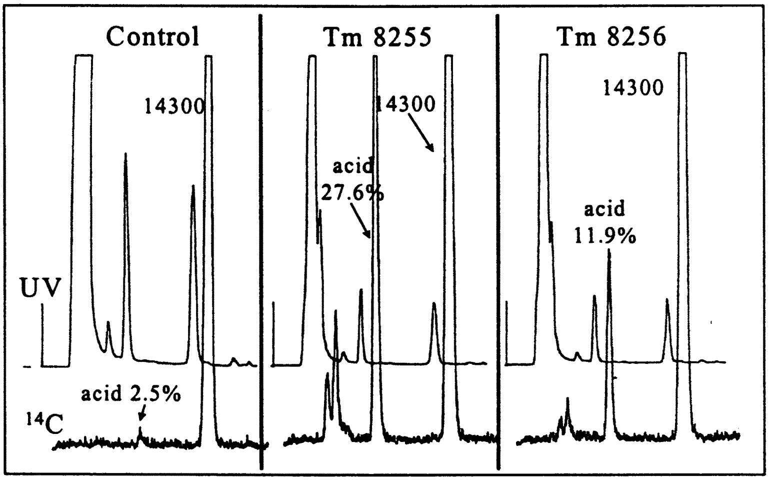


Figure 7. HPLC profiles from UV and ^{14}C detection showing in vivo *hydrolysis of MON 14300 to the acid in control (UC82B) and in two lines of transgenic (8255 and 8256) tomato seedlings (Reproduced from reference 8).*

The fact that RLE3 esterase is expressed in plants and shows *in vitro* activity still does not guarantee that pyridines will be hydrolyzed *in vivo* when applied to plants. Three HPLC profiles illustrating *in vivo* metabolism of MON 14300 in control (UC82B) and two high expressing tomato lines (8255 and 8256) are shown in Figure 6. ^{14}C-MON 14300 was applied to the shoot of 2 day-old seedlings, and 2 days later seedlings were washed with methanol and tissue extracts analyzed. The HPLC chromatograms displayed signals from both the $UV_{254\ nm}$ (upper trace, Figure 7) and radioactivity (lower trace, Figure 7) detectors. For the purpose of metabolite identification, plant extracts were co-injected with a mixture

Downloaded by OHIO STATE UNIV LIBRARIES on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch007

of authentic standards for UV detection. Control tomato seedlings (Figure 7) showed MON 14300 as the primary residue (90.8%) with little acid (2.5%), indicating the lack of endogenous esterase activity against MON 14300. Both transgenic lines (8255 and 8256) showed rapid conversion of MON 14300 to its acid with line 8255 showing greater activity (27.6%) than 8256 (11.9%). These HPLC profiles resulted from extracts of plants 2 days after treatment, and presumably the process of MON 14300 hydrolysis by RLE3 would continue with time. The expression levels of the RLE3 esterase in 8255 and #8256 were at 0.05% and 0.005% of the total protein, respectively. In both transgenic lines small levels of more polar products, likely from further degradation of the acid (*18*), were detected near the solvent front.

Downloaded by OHIO STATE UNIV LIBRARIES on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch007

Assays for Thiazopyr Tolerance in Tobacco and Tomato

Thiazopyr tolerance was evaluated using transgenic tobacco seeds in a root elongation assay. Tobacco R_1 seeds were sterilized and germinated on agar fortified with various concentrations of thiazopyr (0, 0.05, 0.1 and 0.5 μM). With control seedlings, we observed dose-dependent inhibition of root elongation showing about 75% inhibition at 0.1 μM thiazopyr. In comparison, seedlings from a high-expressing line showed normal root length at 0.1 μM thiazopyr with inhibition visible at 0.5 μM.

Tolerance in transgenic tomato was examined in soil with pre-emergence application of thiazopyr. Tomato R_1 seeds from two transgenic lines (8255 and 8256) as well as control UC82B seeds were planted in soil, and thiazopyr was sprayed pre-emergence at rates of 0, 34, 67, 140, 280 g ai/ha. Good germination was observed in all lines and evidence of tolerance was apparent 2-3 weeks later. Relative to unsprayed plants, slight stunting was evident in control plants at 67 g ai/ha. At rates equal to or above 140 g ai/ha, the controls never grew beyond the cotyledonary stage and eventually all died. In comparison, the transgenic lines were normal at 67 g ai/ha and were increasingly stunted at high rates of thiazopyr. The stunted transgenic plants recovered and produced normal flowers, fruits, and seeds.

A more stringent test of plant tolerance employed pre-plant incorporation of thiazopyr. In this method of application, seeds were sown directly into soil which had been incorporated with thiazopyr. Plants were visually rated at 21 days after treatment. Results (Figure 8) showed that at 34 g ai/ha rate, control (UC82B) was slightly injured (75% growth) while the two transgenic lines (8255 and 8230) were normal (100% growth). At 45 g ai/ha, control was nearly dead (10% growth) while the transgenics were nearly normal (90% growth). At 67 g ai/ha, control was dead while the transgenics were stunted (40 to 50% growth). Based on 85% growth inhibition, we estimated the tolerance in transgenic tomato seeds to be 3 times higher than the controls. These results unequivocally demonstrated thiazopyr tolerance in tomato plants expressing the RLE3 esterase.

Discussion

Our efforts to engineer plant tolerance to thiazopyr began with the identification of a non-phytotoxic metabolite (i.e. acid) and selection of an enzyme (i.e. esterase) capable of this catalysis (*5*). We purified a pyridine-esterase, cloned and expressed the cDNA (i.e.

rle3) in plants, and demonstrated hydrolysis of the pyridines *in vitro* and *in vivo*. Tolerance to thiazopyr was demonstrated in transformed tobacco and tomato using a variety of whole plant assays. Our data clearly showed that tolerance was correlated to the expression of RLE3 esterase which catalyzed hydrolysis of thiazopyr. Although plant tolerance was demonstrated only against thiazopyr, tolerance to MON 14300 and MON 2300 is expected to be even greater based on higher rates of hydrolysis by RLE3. Although RLE3 esterase served to validate the concept, we feel an ideal esterase source would be one of microbial origin that has high specificity towards the pyridines and is easily expressed in plants. We believe the level of tolerance can be further optimized by targeting expression to specific tissues and by temporal expression during early germination when exposure to thiazopyr is greatest.

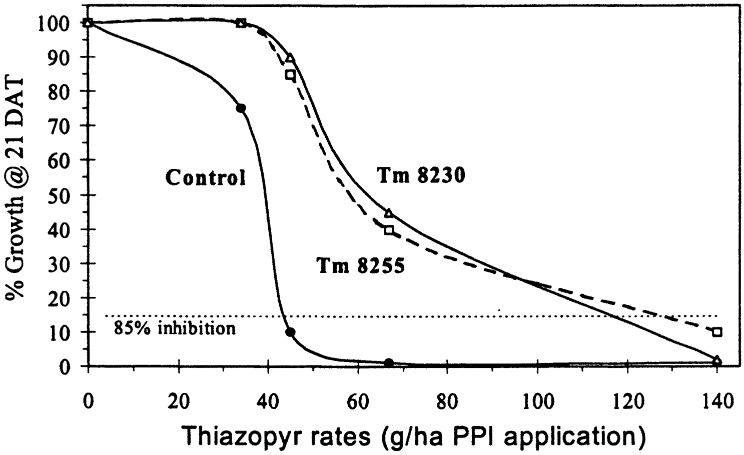


Figure 8. Thiazopyr dose titration under pre-plant incorporation in control and transgenic (8255 and 8230) tomato seeds evaluated at 21 days after seeding.

Aside from engineering thiazopyr tolerance, this work has generated several other interesting observations. The first is the lack of abnormal plant phenotype from constitutive expression of a broad-substrate RLE3 esterase. Whether this is due to targeting of the esterase to the ER or the lack of esterase activity against plant endogenous substrates is not clear. A second observation is the lack of plant endogenous esterase activity against the pyridines in spite of the reported existence of multiple plant esterases (*19, 20, 21*). The role of esterases and mixed function oxygenases in plant de-esterification of xenobiotics is an interesting topic for future research. And finally, our work suggests that a previously published peptide sequence of a rabbit liver esterase (RLE1) may contain errors. This is indicated by the expression of an inactive protein from a cDNA designed based on the published peptide sequence.

Downloaded by OHIO STATE UNIV LIBRARIES on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch007

Acknowledgments

We are grateful to Prof. J. Ozols (Univ. of Connecticut) for providing samples of rabbit liver esterases (isozyme 1 and 2). We also thank technical contributions made by numerous colleagues : T. Solsten for mass spectrometry; S. Rangwala for insect expression, C. Smith for protein sequencing; N. Mathis, J. Layton and A. Howe for plant transformation; S. Rao, W. Kosinsky, B. LaValle, B. Coombs, T. Coomb and G. De Brecht for greenhouse and field support.

Downloaded by OHIO STATE UNIV LIBRARIES on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch007

References

1. Armbruster, B.L.; Molin, W.T.; Bugg, M.W. *Pest. Biochem. Physiol.* **1991**, *39*, 110-120.
2. Armbruster, B.L.; Molin, W.T.; Bugg, M.W. *Plant Physiol. Suppl* **1988**, *86*, 97.
3. Feng, P.C.C.; Solsten, R.T.; McClanahan, R.H. *Pestic. Biochem. Physiol.* **1994**, *48*, 8-14.
4. Feng, P.C.C.; Rao, S.R.; Schafer, D.E. *Pestic. Sci.* **1995**, *45*, 203-207.
5. Feng, P.C.C.; Ruff, T.G.; Kosinski, W.G. *Xenobiotica* **1995**, *25*, 27-35.
6. Feng, P.C.C.; Solsten, R.T. *Xenobiotica* **1994**, *24*, 729-734.
7. Rao, S.R.; Feng, P.C.C.; Shafer, D.E. *Pestic. Sci* **1995**, *45*, 209-213.
8. Feng, P.C.C.; Ruff, T.G.; Rangwala, S.H.; Rao, S.R. *Pest. Biochem. Physiol.* **1998**, *59*, 89-103.
9. Ozols, J. *J Biol Chem.* **1987**, *262*, 15316-15321.
10. Robbi, M.; Beaufay, H.; Octave, J.N. *Biochem J.* **1990**, *269*, 451-458.
11. Horsch, R.B.; Fry, J.E.; Hoffmann, N.L.; Eichholtz; D.; Rogers, S.G.; Fraley, R.T. *Science (Washington, D.C.)* **1985**, *227*, 1229-1231.
12. Guengerich, F.P.; Peterson, L.A.; Bocker, R.H. *J. Biol. Chem.* **1988**, *263*, 8176-8183.
13. Bedford, C.T.; Crayford, J.V.; Hutson, D.H.; Wiggins, E.E. *Xenobiotica* **1978**, *8*, 383-395.
14. Leinweber, F. J. *Drug Metab. Reviews* **1987**, *18*, 379-439.
15. Feng, P.C.C.; Solsten, R.T. *Xenobiotica* **1991**, *21*,1265-1271.
16. Korza, G.; Ozols, J. *J. Biol Chem.* **1988**, *263*, 3486-3495.
17. Ozols, J. *J. Biol Chem.* **1989**, *264*,12533-12545.
18. McClanahan, R.H.; Solsten, R.T.; Feng, P.C.C.; Logusch, E.W. *Pest. Biochem. Physiol.* **1995**, *51*, 90-98.
19. Krell, H.W.; Sandermann, H. *Eur. J. Biochem.* **1984**, *143*, 57-62.
20. Nourse, A.; Schabort, J.C.; Dirr, H.W.; Dubery, I.A. *Phytochem.* **1989**, *28*, 379-383.
21. Brown, H.M.; Wittenbach, V.A.; Forney, D.R.; Strachan, S.D. *Pest. Biochem. Physiol.* **1990**, *37*, 303-313.

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch008

Chapter 8

Biodegradation of Pesticides Containing Carbon-to-Phosphorus Bond

Pawel Kafarski[1], Barbara Lejczak[1], and Giuseppe Forlani[2]

[1]Institute of Organic Chemistry, Biochemistry and Biotechnology, Wroclaw University of Technology, Wybrzeze Wyspianskiego 27, 50–370 Wroclaw, Poland
[2]Department of Genetics and Microbiology, University of Pavia, via Ferrata 1, 27100 Pavia, Italy

Phosphonates constitute a class of organic compounds containing a direct covalent carbon-to-phosphorus bond. They are in widespread use these days, mainly as insecticides, herbicides, antibiotics, lubricants or flame retardants. The fate of phosphonates in the environment attracts considerable attention especially if resistance of the stable C-P bond to chemical, photolytic and thermal cleavage is considered. Organophosphonates are generally considered to be non-persistent because a number of microorganisms provide suitable pathways for the biodegradation of these compounds. Thus, the ability to catabolize phosphonates is widesperad among bacteria and fungi. Metabolic pathways which have been characterized so far are reviewed in this presentation.

Organophosphonates are a group of both synthetic and biogenic compounds characterized by the presence of covalent carbon-to-phosphorus bond. The conversion of phosphonates to phosphate products by living systems, apart from being essential for the return of carbon-bound phosphorus to the phosphate metabolic pool, is of considerable practical importance since phosphonates have recently found extensive application. Compounds containing C-P bond occur in an increasing number of industrial, agricultural, medical and housecleaning products. As a consequence, thousands of tones of these xenobiotics are introduced annually into the environment (*1*). Intensive use of organophosphonate herbicides (glyphosate and glufosinate) and insecticides has raised an increasing concern due to their possible pollution of the environment and stimulated intensive studies on their biodegradation.

Although the C-P bond is resistant to chemical degradation (hydrolytic, thermal or photochemical) (2) organophosphonates are generally considered to be non-persistent

In Pesticide Biotransformation in Plants and Microorganisms; Hall, J., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2000.

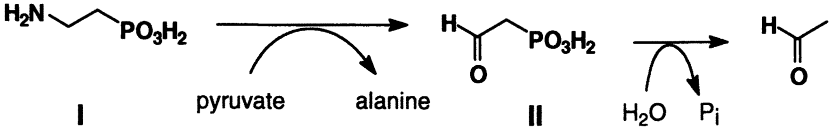
because a number of microorganisms possess pathways suitable for conversion to non-toxic metabolites or complete mineralization of these compounds. Thus, the ability to catabolize phosphonates is widespread among bacteria, and many soilborne strains of *Escherichia, Salmonella, Shigella, Klebsiella, Enterobacter, Serratia, Pseudomonas, Rhizobium, Agrobacterium, Bacillus, Arthrobacter* and *Kluyvera* are able to grow on phosphonates as the sole source of phosphorus (for review see Refs. *3, 4* and *5*). The history of studies on the biodegradation of phosphonates began with the reports of Zeleznick (*6*) and Mastalerz (*7*) on microbial strains capable of growth on various simple phosphonic acids as the sole source of phosphorus. The following thirty-five years of studies resulted in isolation of several hundred bacterial strains capable to split carbon-to-phosphorus bond. On the contrary, surprisingly little is known about the metabolism of these xenobiotics by fungi (*8-12*) although these organisms are supposedly responsible for the biodegradation of organophosphonates in soil. Even though many synthetic organophosphonates may be readily degraded in the environment by biotic transformations, our knowledge of their environmental fate remains limited (*5,13*).

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch008

Catabolism of 2-Aminoethanephosphonic Acid (Ciliatine)

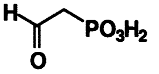
2-Aminoethanephosphonic acid (**I**, AEP) was isolated in 1959 from ciliated sheep rumen protozoa, and thus named ciliatine (*14*). Independently, it was isolated from lower marine animals (*15*). Ciliatine (**I**) is the simplest natural phosphonate and is also the most ubiquitous considering the high levels found in some organisms. Occurrence of AEP is well documented in *Monera, Protista* and animal kingdoms whereas its presence in plants and fungi has not been confirmed (*16*). Therefore, the fact that AEP acid may serve as the sole source of phosphorus for most of the examined microorganisms is not surprising and may be taken as an indication that it is degraded more readily than other phosphonates. The study of AEP utilization by soil microflora has provided useful insight to understand the molecular basis of the catabolism of C-P bond-containing xenobiotics.

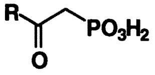
Ciliatine catabolism by many bacteria is a two-step pathway by which this amino phosphonate is ultimately converted to acetaldehyde and orthophosphate (*17-19*). The first step involves a transamination reaction in which the amino group of ciliatine is donated to pyruvate and phosphonoacetaldehyde (**II**) is produced. Phosphonoacetaldehyde is then cleaved by phosphonatase (phosphonoacetaldehyde hydrolase), which exhibits strong substrate specificity towards compound **II**. These two enzymes were isolated from various bacterial sources, mechanisms of their action were thoroughly studied, and the corresponding genes were characterized (*20-28*). Phosphonatase [EC 3.11.1.1] is perhaps the only well-characterized enzyme responsible for carbon-to-phosphorus bond cleavage (*24-26*). Formation of a protonated Schiff base, at a lysine in the active site of the enzyme and the carbonyl group of the substrate, facilitates cleavage of the C-P bond resulting in liberation of acetaldehyde and phosphite.



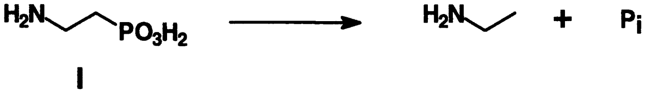
Bacteria capable of metabolizing ciliatine may be divided into two distinct groups. In the first one, represented by *Enterobacter aerogenes* (*5*), *Salmonella typhimurium* (*29*) and *Pseudomonas* sp. (*30*), AEP utilization is regulated by inorganic phosphate, and occurs only when it is the sole source of phosphorus. The second group of bacteria, *Pseudomonas putida* (*31*), *Pseudomonas fluorescens* (*30,33*), *Bacillus cereus* (*18*) and a recently isolated strain of *Streptomyces* sp. (*32*), are not regulated by phosphate concentration and utilize ciliatine as a source of nitrogen, phosphorus and carbon, usually with excretion of inorganic phosphate into the culture media.

2-Oxoalkanephosphonates (III), which are compounds structurally related to phosphonoacetaldehyde, are also readily degraded by bacteria and fungi (*8,9,33*). However, they do not act as substrates or as inhibitors of phosphonatase (Lacoste A.-M., University of Bordeaux, France, private communication). The mechanism of their degradation awaits elucidation.


II


III

Bacterial strains which degrade AEP to orthophosphate and ethylamine by C-P lyase(s) have also been identified (*34,35*). These enzymes catalyze direct cleavage of the carbon-to-phosphorus bond in a wide variety of structurally diverse organophosphonates by a free-radical mechanism that will be discussed later.

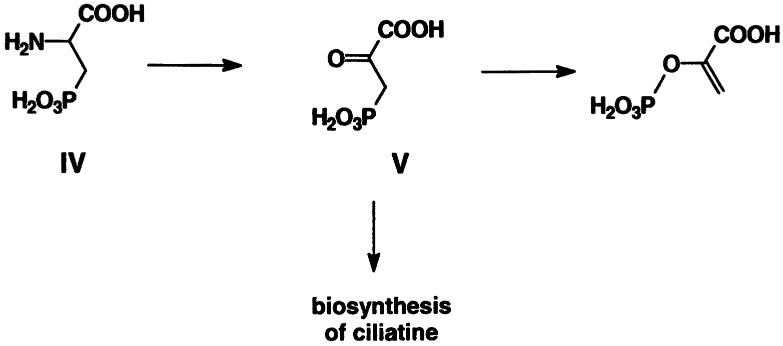


The degradation of AEP by *Protista* has been studied less extensively. Quite surprisingly, no release of inorganic phosphate from ciliatine, amino phosphonate endogenous to *Tetrahymena thermophila*, was ever directly observed *in vivo* in this thoroughly studied organism (*36,37*). Snail eggs contain virtually all stored phosphorus in the form of phosphonates, and during embryonic development the C-P bond is rapidly converted to inorganic phosphate (*38-40*). The mechanisms of this degradation, intermediates, and enzymes involved in this process are unknown.

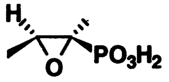
Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch008

Catabolism of Other Naturally Occurring Organophosphonates

The catabolism of other biogenic organophosphonates was studied only incidentally. 2-Amino-3-phosphonopropionic acid (phosphonoalanine, IV) seems to accompany ciliatine in some organisms, but its biological role is completely unknown. The decarboxylation of this amino acid may serve as an alternative pathway for ciliatine synthesis (*41*). Phosphonoalanine is also catabolized through a two-step pathway with initial conversion to phosphonopyruvate (V) with the release of inorganic phosphate. Most likely the cleavage of phosphonopyruvate is catalysed by phosphoenolpyruvate phosphomutase (P-C bond forming enzyme) or a related enzyme (*42-45*). Since phosphonoalanine is a biogenic organophosphonate and its formation is strictly bound to the metabolic pathway of ciliatine, this finding is not surprising.



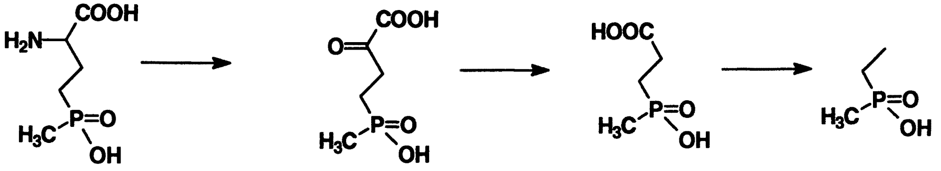
1,2-*cis*-Epoxypropanephosphonic acid (phosphonomycin, VI) is a broad-spectrum antibiotic originally isolated from several strains of streptomycetes (*Streptomyces fradiae*, *S. wedmorensis* and *S. viridochromogenes*) (*46,47*), and later from the bacterium *Pseudomonas syringae* (*48*). It is the first phosphonic acid antibiotic to be used parenterally and is quite widely adopted in human therapy, mostly in Europe (Spain and Italy) and Japan (*49*). Surprisingly there is only one paper devoted to its degradation (*50*). Phosphonomycin is metabolized by a certain strain of *Rhizobium huakuii* through a unique mechanism because this strain was unable to degrade other phosphonates. However, this rhizobial strain utilizes this antibiotic, independent of the cellular phosphate status, as either a carbon source or a carbon and phosphorus source for growth. Degradation is linked with an essentially quantitative release of organophosphonate-derived orthophosphate. Degradation of phosphonomycin by a cleavage of carbon-to-phosphorus bond may represent a new mechanism of bacterial resistance to this antibiotic.



VI

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch008

L-2-Amino-4-[hydroxy(methyl)phosphinyl]butyric acid (phosphinothricin, **VII**), also known as glufosinate, is the active herbicidal ingredient of *Basta®*, produced by Hoechst. This compound is unusual in that it possesses two carbon-phosphorus bonds that make its biodegradation quite difficult. It was at first isolated as part of the antibacterial tripeptide phosphinothricyl-L-alanyl-L-alanine produced by *Streptomyces hygroscopicus* and *S. viridochromogenes* (*51-53*). The tripeptide is highly active *in vitro* against gram-positive and gram-negative bacteria (*49*) and also exerts strong herbicidal properties due to the released phosphinothricin (*54*). Soil studies indicated that phosphinothricin is rapidly metabolized (*55*). The mechanism of its biodegradation is, however, apparently unknown. It was shown that the deamination of the herbicide to the corresponding keto acid (**VIII**) is the first step of this process (*56, 57*). This reaction is (most probably) catalyzed by γ-aminobutyric acid: α-ketoglutarate transaminase, an enzyme involved in utilization of γ-aminobutyric acid (*57*). After prolonged time, decarboxylation of **VIII** occurs with the formation of 3-methylphosphonico-propanoic acid (**IX**) (*56, 58-61*). In soil, a second decarboxylation step results in 2-methylphosphonico-ethanoic acid (**X**) (*62*). In this instance C-P bond cleavage was not observed, and the environmental fate of **X** is unknown (*56*).



VII **VIII** **IX** **X**

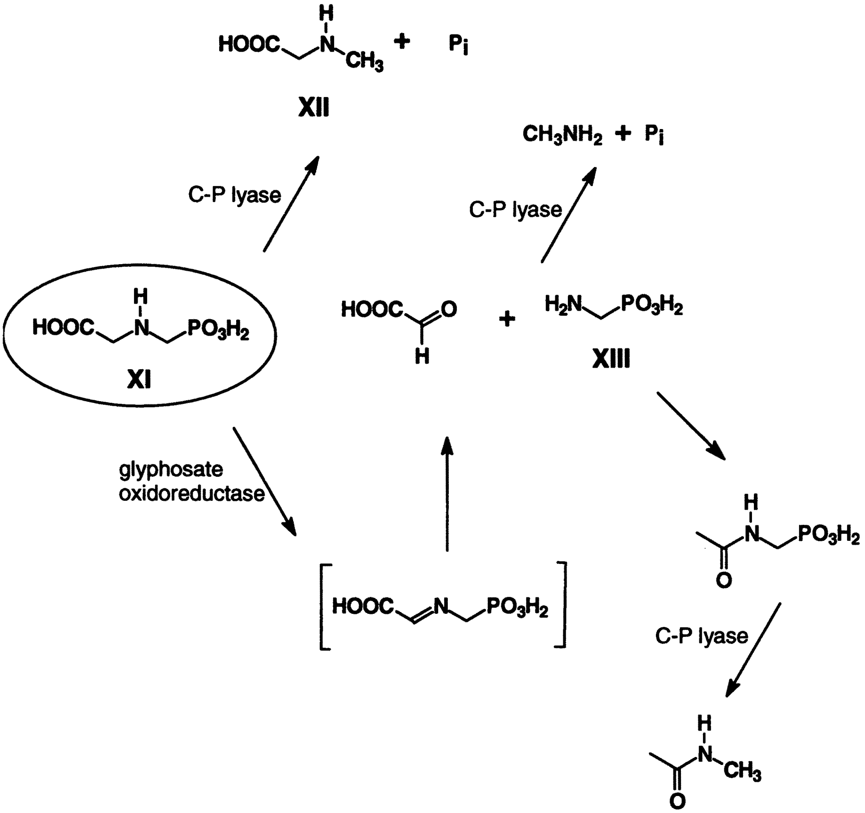
Biodegradation of Glyphosate

The biodegradation of the widely used phosphonate herbicide, *N*-phosphonomethylglycine (glyphosate, **XI**) has been most intensively studied (*3-5*). Glyphosate is the active ingredient of Monsanto's environmentally friendly, broad-spectrum herbicide *Roundup®* (*63*). It has little or no chronic or neuro-toxic effects and no obvious carcinogenic and mutagenic activity. Thus it is being considered to be harmless for man and animals (*63*).

In most cases, glyphosate is rapidly mineralized in the environment (*12, 63-69*). Even though the rate of utilization has been reported to vary considerably between different soils, it is completely degraded by soil microorganisms to water, carbon dioxide and phosphate. Mineralization proceeds without a lag phase and occurs under both aerobic and anaerobic conditions (*63, 66, 69, 70*). An *Achromobacter* strain taken from a glyphosate waste stream treatment facility (*71*), and *Streptomyces* sp. isolated from municipal sewage treatment plant (*32*) are the only microbial strains, reported so far, to be able to utilize the herbicide constitutively in pure culture. This may derive from the fact that the first steps in glyphosate degradation are either accomplished by

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch008

bacterial strains which are quiescent, or proliferate slowly (*72*) or the degradation is mediated by a consortia of microorganisms. Numerous bacterial strains, however, have been described that use glyphosate as a phosphorus source when grown in media without inorganic phosphate. However, the latter was inhibitory to glyphosate breakdown. For example, in a study investigating glyphosate degrading-activity in 163 microbial isolates from the field, 26 isolates were able to metabolize the herbicide to sarcosine (**XII**) and inorganic phosphate under phosphate starvation, but no evidence of its metabolism or co-metabolism to aminomethanephosphonic acid (**XIII**) was obtained (*73*). Sufficient phosphate is usually present in most environments to satisfy microbial nutrition. Thus, it is not surprising that most herbicide metabolism in the soil indicated conversion of glyphosate to aminomethanephosphonic (C-P bond conserved). Glyphosate was degraded in gram-positive and gram-negative bacteria (*3-5,74,75*), and some fungal strains (*9*), by two main pathways, both of which lead to breakage of the carbon-to-phosphorus bond.



In the first pathway, glyphosate is converted to aminomethanephosphonic and glyoxalate (*65, 66, 74, 76-80*) by a flavoprotein, glyphosate oxidoreductase (GOX) (*80, 81*). Aminomethanephosphonic acid is then either directly metabolised to

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch008

methylamine and orthophosphate (*64, 82, 83*), or undergoes acetylation prior to the cleavage of the C-P bond (*80*). The *gox* gene, that encodes glyphosate oxidoreductase, was cloned from *Achromobacter* sp. (*71, 74, 80*). It encodes a 46.1 kDa protein with little homology to other proteins, except for a region near the *N*-terminus that contains a conserved motif associated with flavin binding in flavoenzymes such as D-amino acid oxidases or sarcosine oxidases.

Alternatively, the initial cleavage of the C-P bond yields sarcosine, which is further converted to glycine and a C_1-unit, which is incorporated into purines and some amino acids (*13, 73, 75, 84-88*). The enzyme or enzymes responsible for direct cleavage of organophosphonate C-P bonds are known by the general name "C-P lyase". The activity of this enzyme(s) can be detected in whole organisms; however it has never been convincingly shown in cell-free extracts (*35,86,89-92*).

The uptake and breakdown of phosphonates in *Escherichia coli* is well-characterized genetically (*93-100*). The *phn* gene cluster consists of 17 genes (*phnA* to *-Q*), among which *phnC* to *-P* appear to be required for phosphonate uptake and breakdown. Mutagenesis of the *phn* gene cluster revealed that *phnCDE* encode a phosphonate transporter, *phnF* and *phnO* may have regulatory functions, *phnG* to *-M* are likely to be components of C-P lyase, and *phnN* and p*hnP* are probably accessory proteins. The existence of a homologous system was also demonstrated in *Rhizobium* (*Sinorhizobium*) *meliloti* (*101*), that most probably has a single C-P lyase able to degrade a wide range of phosphonates. It appears to have a broader substrate specificity than its *E. coli* counterpart because *R. meliloti* can grow on glyphosate as sole phosphorus source, while *E. coli* can not. Growth on a similar wide range of phosphonates was also observed in *Agrobacterium radiobacter* (*35*) and *Arthobacter* sp. (*91*), but the broad specificity was proposed to result from activity of two different C-P lyases.

Rhizobium meliloti (*101*) and *Enterobacter aerogenes* (*29*) are also examples of microorganisms having two or more enzymes responsible for breakage of C-P bond. The second enzyme seems to be phosphonatase, since disruption of the *phn* gene cluster in these organisms does not prevent their growth on ciliatine.

C-P Lyase Mediated Biodegradation of Other Synthetic Phosphonates

The microbial utilization of a wide variety of synthetic phosphonates has been extensively studied to date. In most described cases, microorganisms utilize organophosphonates as a sole source of phosphorus, and thus it is believed that the cleavage of C-P bond is catalyzed by C-P lyase. This enzyme is unusual in that it hydrolyzes this bond even in simple substrates such as alkyl- and arylphosphonates (*34,102-105*). For example, *Agrobacterium radiobacter* was found to cleave C-P bond of alkyl-, vinyl, propenyl-, propynyl- phenyl- and chloromethyl-phosphonic acids, yielding upon hydrolysis alkanes, ethylene, propylene, propyne (acetylene), benzene and chloromethane (*35*). Thus, this microorganism degrades organophosphonates in which phosphorus atom is bound to carbon atoms of diverse electronic properties with all the possible carbon atom hybridizations involved (*sp^3*, *sp^2* and *sp*). The structural diversity of substrates being cleaved by various microorganisms is quite striking. For

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch008

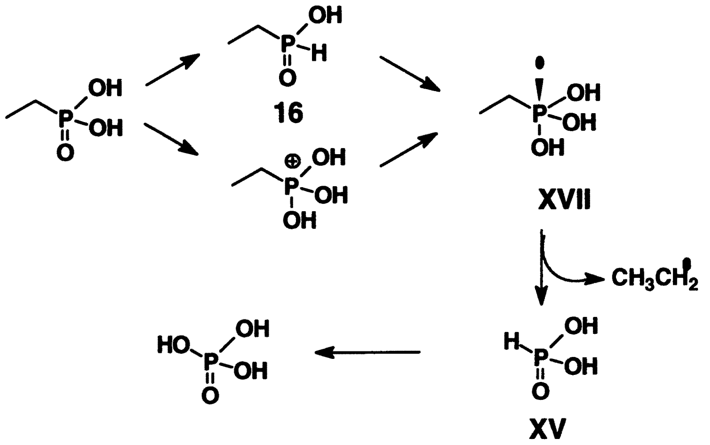
Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch008

example, *Penicillium notatum* degraded 13 out of 16 phosphonates (mostly aminophosphonates) under phosphorus-starvation (*9*), and some were apparently metabolized by C-P lyase. A wild-type strain of *Streptomyces* sp. grew on 8 of 9 aminophosphonates (including glyphosate and ciliatine), and on 6 of 7 other phosphonates (including methanephosphonic acid, phosphonoacetic acid and phosphonomycin) (*32*).

C-P lyase has been assayed by measuring the release of methane from methanephosphonic acid by whole cells (*103-106*). The details of the reaction mechanism are largely not understood, although the reaction involves direct cleavage of the phosphonate bond without prior activation. However, biochemical investigations have been hindered by the lack of a reliable assay for C-P lyase activity in cell-free extracts, and thus studies have concentrated either on chemical modelling of this reaction, or cloning of the genes involved in phosphonate degradation.

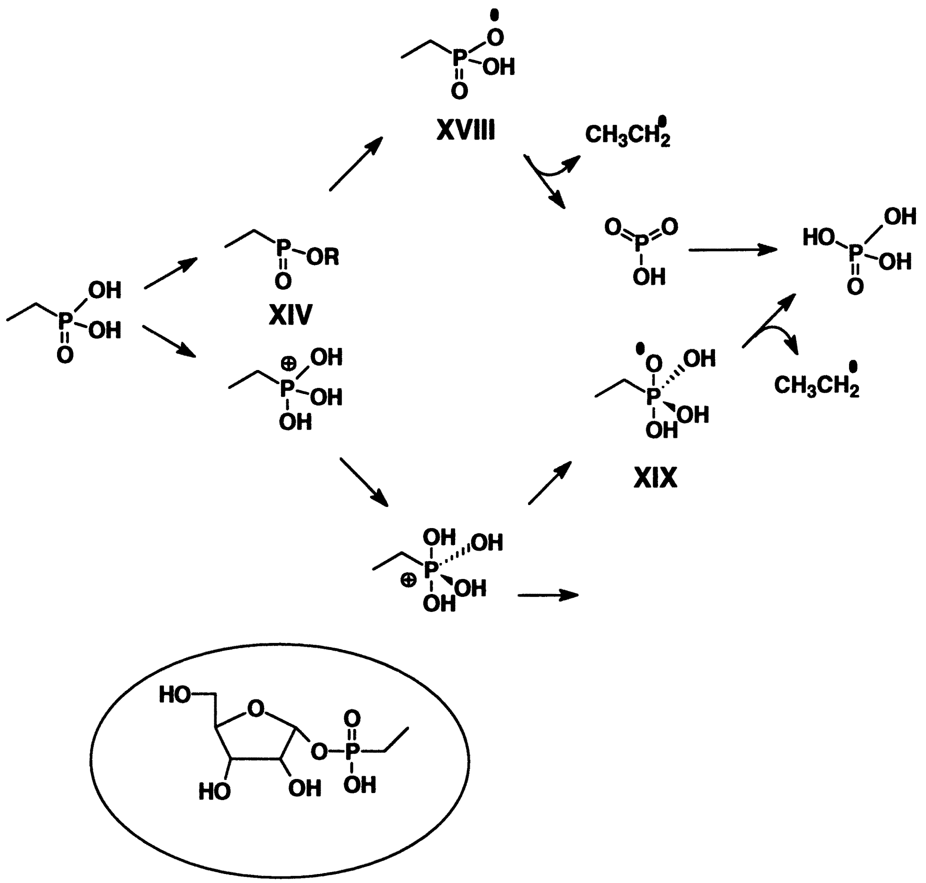
Carbon fragments generated from alkanephosphonic acids during microbial cleavage of C-P bonds have been postulated to reflect the formation of radical intermediates in this process. Results of chemical modelling based upon oxidation with lead (IV) tetraacetate (*103*) and the photo-assisted Fenton reaction [oxidation with Fe(III)/H_2O_2] (*107*) supported this hypothesis. Two possible pathways of alkanephosphonate biodegradation were thus postulated in which organophosphoranyl (*95,108*) or organophosphonyl (*103,104,109*) radicals are formed.

Phosphonyl intermediate



Studies of *Escherichia coli* (*110*) utilization of [^{32}P]-ethylphosphonic acid, identified formation of α-1-ethylphosphonoribose (**XIV**) in extracellular culture supernatant. Mutation of the genetic locus, which is essential to C-P bond cleavage, also disrupted organophosphonate ribosylation. This observation, alongside with the absence of phosphorous acid (phosphite, **XV**) and ethanephosphonous acid (**XVI**) in the growth medium favours organophosphoranyl (**XVII**) as an intermediate. However, organophosphonyl radical (**XVIII** or **XIX**) intermediacy may still be possible, because neither labelled inorganic phosphate nor ethane were detected when α-1-ethylphosphonoribose was incubated with *E. coli* cell lysate.

Phosphoranyl intermediate



Studies of *Escherichia coli* (*110*) utilization of [^{32}P]-ethylphosphonic acid, identified formation of α-1-ethylphosphonoribose (**XIV**) in extracellular culture supernatant. Mutation of the genetic locus, which is essential to C-P bond cleavage, also disrupted organophosphonate ribosylation. This observation, alongside with the absence of phosphorous acid (phosphite, **XV**) and ethanephosphonous acid (**XVI**) in the growth medium favours organophosphoranyl (**XVII**) as an intermediate. However, organophosphonyl radical (**XVIII** or **XIX**) intermediacy may still be possible, because neither labelled inorganic phosphate nor ethane were detected when α-1-ethylphosphonoribose was incubated with *E. coli* cell lysate.

Genetic studies have provided additional biological evidence considering the mechanism of dephosphonylation by C-P lyase *in vivo*. Mutants that have lost the ability to utilize phosphonates as sole source of phosphorus, simultaneously lose the

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch008

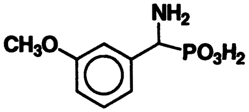
ability to assimilate phosphorous acid (phosphite) (*97*). Complementation of one phenotype led without exception, to restoration of the other as well (*97*). Thus, it has been proposed that phosphonate degradation yields phosphite (either free or enzyme-bound) as the initial product of the reaction, followed by phosphite oxidation to inorganic phosphate (*97,111,112*). This constitutes the first biological mechanistic evidence for phosphonate degradation by C-P lyase and favours organophosphonyl radicals as intermediates (**XVI** or **XVII**) in the process.

These observations provided additional support if considering the mechanism of action of fungicidal Fosetyl-Al (aluminium tris-O-ethyl phosphonate) and its analogues (*113-115*). These systemic fungicides are taken up and hydrolyzed within the plant, with the release of phosphite, which is thought to act directly on the invading pathogen (*Phytophtora*) by substantial disruption of phosphorus metabolism (*116-118*).

The apparent involvement of a membrane component in C-P lyase-catalyzed degradation of phosphonates was shown (*97*). This is not surprising since it represents a common feature for enzymes that carry out redox reactions. This may also explain the lack of demonstrable *in vitro* C-P lyase activity, since cellular disruption methods would invariably destroy membrane-associated complexes.

Chemical modelling indicated that dephosphonylation of aminoalkylphosphonic acids may also occur as a spontaneous side-reaction after the condensation of aminophosphonate with pyridoxal 3'-phosphate (*119*). Although it represents an unlikely mechanism for C-P lyase, existence of such a pathway should not be ruled out if one considers the degradation of amino(3-methoxyphenyl)methanephosphonic acid (**XX**) in many systems (*9,11,32,33*). The demonstration of the involvement of this reaction, however, will require additional study.

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch008

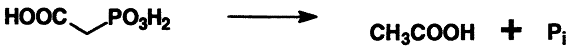


XX

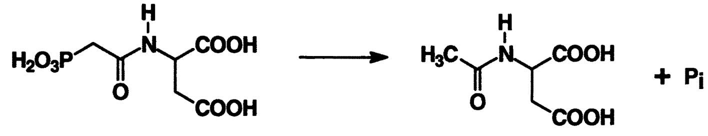
Phosphonoacetate Biodegradation

Phosphonoacetate hydrolase is the third enzyme which catalyzes cleavage of carbon-phosphorus bond. This inducible, zinc dependent enzyme composed of two identical subunits was isolated from *Pseudomonas fluorescens* grown on phosphonoacetate (**XXI**) as sole carbon and phosphorus source (*120-123*). It catalyzes the cleavage of the latter compound to acetate and inorganic phosphate, and represents an alternative route for the microbial metabolism of phosphonates.

The gene encoding phosphonoacetate hydrolase has been cloned, sequenced and expressed in both *Pseudomonas putida* and *Escherichia coli* (*124*). Neither the nucleotide nor the deduced amino acid sequence showed homology with any gene coding for a C-P cleaving enzyme analysed so far.



XXI



XXII XXIII

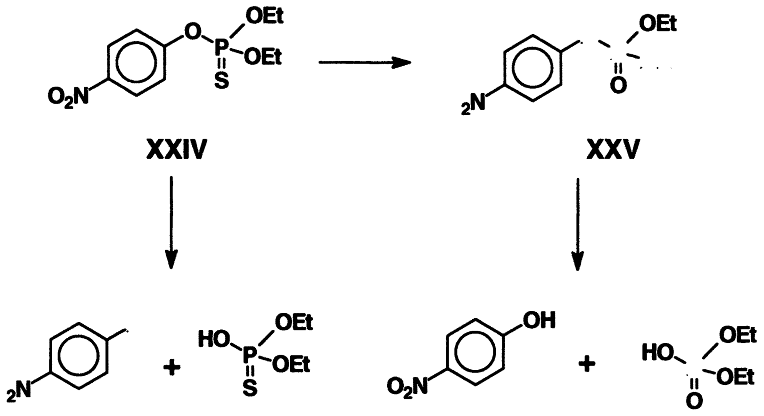
Helicobacter pylori is resistant to the toxic action of *N*-phosphonoacetyl-L-aspartate (**XXII**) due to a similar hydrolase (*125*). Once inside the bacterial cell, this potent inhibitor of the second step of pyrimidine biosynthesis (*126*) was most likely catabolized by phosphonoacetate hydrolase yielding *N*-acetyl-L-aspartate (**XXIII**). The enzyme from *H. pylori* also cleaved phosphonoacetate (*125*). It is also important to note that a recently isolated strain of *Streptomyces* sp. was found to utilize phosphonoacetate as sole source of carbon and phosphorus for growth, suggesting that it also may possess this enzyme (*32*).

Biodegradation of Organophosphonate Insecticides

Initial steps of bacterial degradation of organophosphonate insecticides and nerve agents involve hydrolysis of these organophosphorus esters. This ability appears to be widespread in the environment, and a variety of bacterial species have been isolated which can utilize these compounds (*127-132*). Biodegradation of diethyl *p*-nitrophenyl phosphate (parathion, **XXIV**) has been most intensively studied (*128-130,133*) and results show that hydrolysis of its arylphosphonate moiety is catalysed by phosphotriesterase (aryldialkylphosphatase, EC 3.1.8.1). In insects and other higher organisms, parathion is rapidly converted by monooxygenases (most possibly cytochrome P-450) to its phosphate homologue (paraoxon, **XXV**) which is responsible for the toxic effect of pesticide (*133,134*). Paraoxon is also readily hydrolysed by phosphotriesterase (*134*).

In bacteria this oxidative reaction is only a minor pathway (*133*). Phosphotriesterase has been purified from several bacterial species (*134-140*). In some of them it is a membrane-associated enzyme, whereas in others it is cytosolic. It catalyses a single hydrolysis of dialkyl aryl phosphates and alkyl aryl phosphonates to their corresponding alkyl esters or free acids by a mechanism in which water attack on phosphorus is facilitated by collaborative action of two metal ions (Zn^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+} or Cd^{2+}) present in its active site. Phosphotriesterase accepts a broad range of substrates. Genes encoding organophosphonate degrading-enzymes have been cloned and sequenced in *Flavobacterium* sp. (*137,138,140-142*).

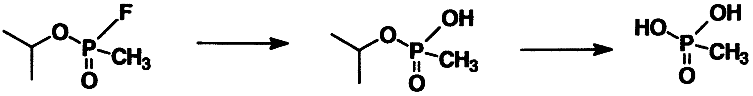
Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch008



XXIV XXV

Degradation of parathion to *p*-nitrophenol and diethyl phosphorothionate reduces mammalian toxicity by a factor of 122. Since most interest has been directed towards detoxification, studies on the ultimate metabolic fate of the C-P products have not been extensive. Phosphomonoesterases and phosphodiesterases, which degrade methyl and dimethyl phosphate have been reported in *Klebsiella aerogenes* (*143*).

Organophosphorus acid hydrolase (EC 3.1.8.2) isolated from the thermophilic bacterium *Alteromonas* sp., displayed high hydrolytic activity towards the nerve agents 2-(3,3-dimethylbutyl) methanephosphonofluoridate (soman) and isopropyl methanephosphonofluoridate (sarin, **XXVI**) which contain P-F bond in their chemical structure (*144-148*). The hydrolytic action of this enzyme is similar to that reported for phosphotriesterase.



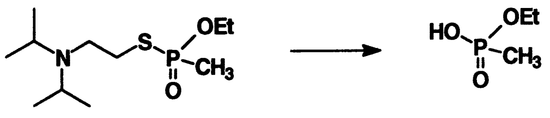
XXVI XXIX XXX

The P-S bond in certain phosphorothiolates, such as ethyl 2-(*N*,*N*-diisopropylamino)ethylthio methanephosphonate (nerve agent VX, **XXVII**) and its insecticidal analogue diethyl 2-(*N*,*N*-diisopropylamino)ethylthio phosphate (Amiton, **XXVIII**), is usually more resistant to enzymatic hydrolysis, and thus, they are less readily biodegradable. However, it has been recently shown that degradation of this compound was easily achieved by the action of white rot fungus *Pleurotus ostreatus* laccase (phenol oxidase) used together with the mediator 2,2'-azinobis(3-ethylbenzylthiazoline-6-sulfonate) (*149*).

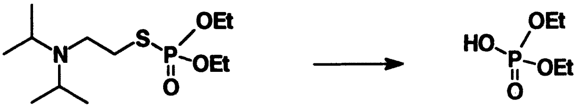
Biodegradation of carbon-to-phosphorus bond in products of the detoxification of organophosphonate insecticides and nerve agents has been studied only incidentally. A good example is a research on complete biodegradation of sarin metabolite (**XXIX**) by mixed cultures of bacteria which carried out sequential hydrolysis of the metabolite to

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch008

methanephosphonic acid (**XXX**) with further degradation of the carbon-to-phosphorus bond (*150*). Although enzyme analysis was not conducted in this research, the involvement of C-P lyase in the final step of degradation was postulated.



XXVII



XXVIII

Conclusions

Several enzymatic systems have been discussed that are involved in the rapid utilization of organophosphonates by soil microflora. However, phosphonatase (EC 3.11.1.1) is the only well-characterized hydrolytic enzyme to date. Moreover, even though the first catabolic products have been identified in most cases, their environmental fate is still far from being fully elucidated. It is also important to note that there is a lack of C-P bond degrading enzyme(s) in plants. Compounds containing carbon-phosphorus bonds are finding increasing use in many different applications. Therefore, biodegradation studies are urgently required to ensure that their usage does not give rise to serious environmental contamination, and to develop efficient and specific biological waste treatment systems using microorganisms especially selected for these individual xenobiotics. In the future, the neglected study of the role of fungal species in such research might provide useful enzymes or enzyme systems for the bioremediation of C-P compounds. Several microbial processes also show promise for inactivating and degrading dangerous warfare agents containing C-P bonds. However, attempts to use isolated microorganisms, as well as bacterial consortia to hydrolyze C-P bond have been unsuccessful so far (*151*).

References

1. Egli, T. *Microbiol. Sci.* **1988**, *5*, 36-41.
2. Freedman, L. D.; Doak, G. O. *Chem. Rev.* **1957**, *57*, 479-523.
3. Ghisalba, O.; Küenzi, M.; Ramos Tombo, G.; Schär M. *Chimia* **1987**, *41*, 206-215.

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch008

4. Kertesz, M. A.; Cook, A. M.; Leisinger, T. *FEMS Microbiol. Rev.* **1994**, *15*, 195-215.
5. Ternan, N. G.; Mc Grath, J. W.; Mc Mullan, G.; Quinn J. P. *World J. Microbiol. Biotechnol.* **1999**, *14*, 635-647.
6. Zeleznick, L. D.; Meyers, T. C.; Titchener, E. B. *Biochim. Biophys. Acta* **1963**, *78*, 546-547.
7. Mastalerz, P.; Wieczorek, Z.; Kochman, M. *Acta Biochim. Pol.* **1965**, *12*, 151-156.
8. Zboinska, E.; Maliszewska, I.; Lejczak, B.; Kafarski, P. *Lett. Appl. Microbiol.* **1992**, *15*, 269-272.
9. Bujacz, B.; Wieczorek, P.; Krzysko-Łupicka, T.; Golab, Z.; Lejczak, B.; Kafarski, P. *Appl. Environ. Microbiol.* **1995**, *61*, 2905-2910.
10. Sobera, M.; Wieczorek, P.; Lejczak, B.; Kafarski, P. *Toxicol. Environ. Chem.* **1997**, *61*, 229-235.
11. Krzysko-Lupicka, T.; Strof, W.; Kubs, K.; Skorupa, M.; Wieczorek, P.; Lejczak, B.; Kafarski, P. *Appl. Microbiol. Biotechnol.* **1997**, *48*, 549-552.
12. Krzysko-Lupicka, T.; Orlik A. *Chemosphere* **1997**, *34*, 2601-2605.
13. Schowanek, D.; Verstraete, W. *Appl. Environ. Microbiol.* **1990**, *56*, 2501-2510.
14. Horiguchi, M.; Kandatsu, M. *Nature* **1959**, *184*, 901-902.
15. Kittredge, J. S.; Roberts, E.; Simonsen, D. G. *Biochemistry* **1962**, *1*, 624-628.
16. Kafarski, P.; Mastalerz, P. *Aminophosphonates: Natural Occurence, Biochemistry and Biological Properties*; Oehme, P. H., Lowe, H., Gores, E. Eds.; Beitrage für Wirkstofforschung 1984 Institut fur Wirkstofforschung, Berlin, Vol. 21.
17. Rosenberg, H.; La Nauze, J. M. *Biochim. Biophys. Acta* **1967**, *141*, 79-90.
18. La Nauze, J. M.; Rosenberg, H. *Biochim. Biophys. Acta* **1968**, *165*, 438-447.
19. Cassaigne, A.; Lacoste, A.-M.; Neuzil, E. *Compt. Rend. Hebd. Seances Acad. Sci.* **1969**, *269D*, 254-257.
20. Dumora, C.; Lacoste, A.-M.; Cassaigne, A. *Eur. J. Biochem.* **1983**, *133*, 119-125.
21. Lacoste, A.-M.; Dumora, C.; Balas, L.; Hammerschmidt, F.; Vercauteren, J. *Eur J. Biochem.* **1993**, *215*, 841-844.
22. La Nauze, J.; Rosenberg, M.; Shaw, D. C. *Biochim. Biophys. Acta* **1970**, *212*, 332-350.
23. Dumora, C.; Lacoste, A.-M.; Cassaigne, A. *Biochim. Biophys. Acta* **1989**, *997*, 193-198.
24. Olsen, D. B.; Hepburn, T. W.; Moos, M.; Mariano, P. S.; Dunaway-Mariano, D. *Biochemistry* **1988**, *27*, 2229-2234.
25. Olsen, D. B.; Hepburn, T. W.; Lee, S.-L.; Martin, B. M.; Mariano, P. S.; Dunaway-Mariano, D. *Arch. Biochem. Biophys.* **1992**, *296*, 144-151.
26. Lee, S.-L; Hepburn, T. W.; Swartz, W. H.; Ammon, H. L.; Mariano, P. S.; Dunaway-Mariano, D. *J. Am. Chem. Soc.* **1992**, *114*, 7346-7354.
27. Jiang, W.; Metcalf, W. W.; Lee, K.-I.; Wanner, B. L. *J. Bacteriol.* **1995**, *177*, 6411-6421.
28. Dumora, C.; Marche, M.; Doignon, F.; Aigle, M.; Cassaigne, A.; Crouzet, M. *Gene* **1997**, *197*, 405-412.
29. Lee, K. S.; Metcalf, W. W.; Wanner, B. L. *J. Bacteriol.*, **1992**, *174*, 2501-2510.

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch008

30. Mc Mullan, G.; Watkins, R.; Harper, D. B.; Quinn, J. P. *J. Biochem.* **1992**, *25*, 271-279.
31. Ternan, N. G.; Quinn, J. P. *System. Appl. Microbiol.* **1998**, *21*, 346-352.
32. Obojska, A.; Lejczak, B; Kubrak, M. *Appl. Microbiol. Biotechnol.* **1999**, *51*, 872-876.
33. Zboińska E.; Lejczak, B; Kafarski, P. *Appl. Environ. Microbiol.* **1992**, *58*, 2993-2999.
34. Avila L. Z.; Loo, S. H.; Frost, J. W. *J. Am. Chem. Soc.* **1987**, *109*, 6758-6764.
35. Wackett, L. P.; Shames, S. L.; Venditti, C. P.; Walsh, C. T. *J. Bacteriol.* **1987**, *169*, 710-717.
36. Smith, J. D.; O'Malley, M. N. *Biochim. Biophys. Acta* **1978**, *528*, 394-398.
37. Smith, J. D.; Giegel, D. A. *Arch. Biochem. Biophys.* **1981**, *206*, 420-423.
38. Meneses. P.; Henderson. T. O.; Glonek T. *J. Am. Osteopath. Ass.* **1986**, *86*, 667.
39. Miceli, M. V.; Henderson, T. O.; Myers, T. C. *Am. J. Trop. Med. Hyg.* **1987**, *36*, 355-360.
40. Meneses, P.; Glonek, T. O.; Henderson, T. O. *Comp. Biochem. Physiol.* **1987**, *88B*, 969-975.
41. Warren, W. A. *Biochim. Biophys. Acta* **1968**, *156*, 340-346.
42. Nakashita H.; Shimazu, A.; Seto, H. *Agric. Biol. Chem.* **1991**, *55*, 2825-2829.
43. Nakashita H.; Shimazu, A.; Hidalk, T.; Seto, H. *J. Bacteriol.* **1992**, *174*, 6857-6861.
44. Ternan, N. G.; Quinn, J. P. *Biochem. Biophys. Res. Commun.* **1998**, *248*, 378-381.
45. Ternan, N. G.; McGrath, J. W.; Quinn, J. P. *Appl. Environ. Microbiol.* **1998**, *64*, 2291-2294.
46. Hendlin, D.; Stapley, E. O.; Jackson, M.; Wallik, H.; Miller, A. K.; Wolf, F. J.; Miller, T. W.; Chaiet, L.; Kahan, F. M.; Foltz, E. L.; Woodruff, H. B.; Mata, J. M.; Hernandez, S.; Mochales, S. *Science* **1969**, *166*, 122-123.
47. Stapley, E. O.; Hendlin, D.; Mata, J. M.; Jackson, M.; Wallick, H.; Hernandez, S.; Mochales, S.; Currie, S. A.; Miller, R. M. *Ann. Inst. Farmacol. Espan.* **1970**, *17-18*, 421-434.
48. Shoji, J.; Kato, T.; Hinoo, H.; Hattori, T.; Hirooka, K.; Matsumoto, K.; Tanimoto, T.; Kondo, E. *J. Antibiot.* **1986**, 39, 1011-1012.
49. Uri, J. V.; Bendas, C. M.; Burdash, N. *Acta Microbiol. Hung.* **1988**, *35*, 221-257.
50. McGrath J. W.; Hammerschmidt, F.; Quinn, J. P. *Appl. Environ. Microbiol.* **1998**, *64*, 356-358.
51. Bayer, E.; Gugel, K. H.; Hägele, K.; Hagenmaier, H.; Jessipow, S.; König, W. A.; Zähner, H. *Helv. Chim. Acta* **1972**, *55*, 224-239.
52. Ogawa, Y.; Yoshida, M.; Inouye, S.; Niida, T. *Sci. Reports Meiji Seika Kaisha* **1973**,*13*, 54-59.

53. Kondo, Y.; Shomura, T.; Ogawa, Y.; Tsuruoka, T.; Watanabe, H.; Tutsukawa, K.; Suzuki, T.; Moriyama, C.; Inouye, S.; Niida, T. *Sci. Reports Meiji Seika Kaisha* **1973**, *13*, 34-41.
54. Tachibana, K. *J. Pestic. Sci.* **1986**, *11*, 33-37.
55. Götz, W.; Dorn, E.; Ebert E.Leist, K.-H.; Köcher, H. *Asian Pacific Weed Sci. Soc. 9th Conference*, Manila **1993**, 401-404.

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch008

56. Tebbe, C. C.; Reber, H. H. *Appl. Microbiol. Biotechnol.* **1988**, *29*, 103-105.
57. Schulz, A.; Taggeselle, P.; Tripier, D.; Bartsch, K. *Appl. Environ. Microbiol.* **1990**, *56*, 1-6.
58. Tebbe, C. C.; Reber, H. H. *Biol. Fertil. Soils* **1991**, *11*, 62-67.
59. Bartsch, K.; Tebbe, C. C. *Appl. Environ. Microbiol.* **1989**, *55*, 711-716.
60. Smith, A. E. *J. Agric. Food Chem.* **1989**, *37*, 267-271.
61. Gallina, M. A.; Stephenson, G. R. *J. Agric. Food Chem.* **1992**, *40*, 165-168.
62. Behrendt, H.; Matthies M.; Gildemeister, H.; Görlitz, G. *Environ. Toxicol. Chem.* **1990**, *9*, 541-549.
63. *The Herbicide Glyphosate*, Grossbard, E., Atkinson, D., Eds.; Butterworth, London 1985.
64. Rueppel M. L.; Brighthwell, B. B.; Schaefer, J.; Marvel, J. T. *J. Agric. Food Chem.* **1977**, *25*, 517-528.
65. Nomura, N. S.; Hilton, H. W. *Weed Res.* **1977**, *17*, 113-121.
66. Sprankle, P.; Meggitt W. F.; Penner, D. *Weed Sci.* **1975**, *23*, 229-234.
67. Malik, J.; Barry, G.; Kishore, G. M. *Biofactors* **1989**, *2*, 17-25.
68. Zaranyika, M. F.; Nyandoro M. G. *J. Agric. Food Chem.* **1993**, *41*, 838-841.
69. Cheah,U.-B.; Kirkwood, R. C.; Lum, K.-Y. *J. Agric. Food Chem.*, **1998**, *46*, 1217-1223.
70. Torstensson N. T. L.; Aamisepp, A. *Weed Res.* **1977**, *17*, 209-211.
71. Barry, G. F.; Kishore, G. M.; Padgette, S.; Taylor, S.; Kolacz, K.; Weldon, M.; Re, D. B.; Eichholtz, D. E.; Fincher, K.; Halla, L. In *Biosynthesis and Molecular Regulation of Amino Acids in Plants*, Singh, B. K., Flores, H. E., Shannon, J. C. Eds.: American Society of Plant Physiology, Rockville, 1992, pp. 139-145.
72. Forlani, G.; Mangiagalli, A.; Nielsen, E.; Suardi, M. C. *Soil. Biol. Biochem.* **1999**, *31*, 991-997.
73. Dick, R. E.; Quinn, J. P. *Appl. Microbiol. Biotechnol.* **1995**, *43*, 545-550.
74. Hallas, L. E.; Hahn, E. M.; Korndorfer C. *J. Industrial Microbiol.* **1988**, *3*, 377-385.
75. Quinn, J. P.; Peden, J. M. M.; Dick, R. E. *Appl. Microbiol. Biotechnol.* **1989**, *31*, 283-287.
76. Balthazor, T. M.; Hallas, L. E. *Appl. Environ. Microbiol.* **1986**, *51*, 432-434.
77. Jacob, G. S.; Garbow, J.; Hallas, L. E.; Kishore, G. M.; Schaefer J. *Appl. Environ. Microbiol.* **1988**, *54*, 2952-2958.
78. Pipke, R.; Amrhein, N. *FEMS Microbiol. Lett.* **1988**, *236*, 135-138.
79. Mc Auliffe, K. S.; Hallas, L. E.; Kulpa, C. F. *J. Ind. Microbiol.* **1990**, *6*, 219-221.
80. Kishore, G. H.; Barry, G. *International Patent WO 92/00377 (PTC/US91/04 514)* **1992**.

81. Carson, D. B.; Heitkamp, M. A.; Hallas, L. E. *Can. J. Microbiol.* **1997**, *43*, 97-101.
82. Cook, A. M.; Daughton, C. G.; Alexander, M. *J. Bacteriol*, **1978**, *133*, 85-90.
83. Harkness, D. R. *J. Bacteriol.* **1966**, *92*, 623-627.
84. Jacob, G. S.; Schaefer J.; Stejskal, E. O.; McKay R. A. *J. Biol. Chem.* **1985**, *260*, 5899-5904.
85. Kishore, G. H.; Jacob, G. S. *J. Biol. Chem.* **1987**, *262*, 12164-12168.

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch008

86. Pipke, R.; Amrhein, N.; Jacob, G. S.; Kishore, G. M. *Eur. J. Biochem.* **1987**, *165*, 267-273.
87. Quinn, J. P.; Peden, J. M. M.; Dick, R. E. *Appl. Microbiol. Biotechnol.* **1988**, *29*, 511-516.
88. Dick, R. E.; Quinn, J. P. *FEMS Microbiol. Lett.* **1995**, *134*, 177-182.
89. Daughton, C. G.; Cook, A. M.; Alexander, M. *J. Agric. Food Chem.* **1979**, *27*, 1375-1382.
90. Moore, I. K.; Braymer, H. D.; Larson, A. D. *Appl. Environ. Microbiol.* **1983**, *46*, 316-320.
91. Kertesz, M.; Elgorriaga, A.; Amrhein, N. *Biodegradation* **1991**, *2*, 53-66.
92. Wanner, B. L. *Biodegradation* **1994**, *5*, 175-184.
93. Wanner, B. L.; McSharry, R. J. *Mol. Biol.* **1982**, *158*, 347-363.
94. Wackett, L. P.; Venditti, C. P.; Walsh, C. T.; Wanner, B. L. *J. Bacteriol.* **1987**, *169*, 1753-1756.
95. Wanner, B. L.; Boline J. A. *J. Bacteriol.* **1990**, *172*, 1186-1196.
96. Chen, C. M.; Ye, Q. Z.; Zhu, Z. M.; Wanner, B. L.; Walsh, C. T. *J. Biol. Chem.* **990**, *265*, 4461-4471.
97. Metcalf, W. W.; Wanner, B. L. *J. Bacteriol.* **1991**, *173*, 587-600.
98. Wanner, B. L.; Metcalf, W. W. *FEMS Microbiol. Lett.* **1992**, *100*, 133-139.
99. Makino, K.; Kim, S.; Shinagawa, H.; Amemura, M.; Nakata, A. *J. Bacteriol.* **1991**, *173*, 2665-2672.
100. Kim, S. K.; Makino, K.; Amemura, M.; Shinagawa, H.; Nakata, A. *J. Bacteriol.* **1993**, *175*, 1316-1324.
101. Parker, G. F.; Higgins, T. P.; Hawkes, T.; Robson, R. L. *J. Bacteriol.* **1999**, *181*, 389-395.
102. Daughton , C. G.; Cook, A. M.; Alexander, M. *FEMS Microbiol. Lett.* **1979**, *134*, 177-182.
103. Cordeiro, M. L.; Pompliano, D. L.; Frost, J. W. *J. Am. Chem. Soc.* **1986**, *108*, 332-334.
104. Frost, J. W; Loo, M. L.; Cordeiro, M. L.; Li, D. *J. Am. Chem. Soc.* **1987**, *109*, 2166-2171.
105. Shames, S. L.; Wackett, L. P.; LaBarge, S.; Kuczkowski R. L.; Walsh, C. T. *Bioorg. Chem.* **1987**, *15*, 366-373.
106. Ahn, Y. H.; Ye, Q. Z.; Cho, H. J.; Walsh, C. T.; Floss, H. G. *J. Am. Chem, Soc.* **1992**, *114*, 7953-7954.
107. Huston, P.; Pignatello, J. J. *Water Res.* **1999**, *33*, 1238-1246.
108. Avila L. Z.; Frost, J. W. *J. Am. Chem. Soc.* **1989**, *111*, 8969-8970.
109. Avila, L. Z.; Frost, J. W. *J. Am. Chem. Soc.* **1988**, *110*, 7904-7905.
110. Avila, L. Z.; Draths, K. M.; Frost, W. J. *Bioorg. Med. Chem. Lett.* **1991**, *1*, 51-54.
111. Ohtake, H.; Wu, H.; Imazu, K.; Anbe, Y.; Kato, J.; Kuroda, A. *Resour. Conserv. Recycl.* **1996**, *18*, 125-134.
112. Imazu, K.; Tanaka, S.; Kuroda, A.; Anbe, Y.; Kato, J.; Ohtake, H. *Appl. Environ. Microbiol.* **1998**, *64*, 3754-3758.
113. Williams D.J.; Beach, B. G. W.; Horriere D.; Marachel, G. *Proc. Brit. Crop Prot. Conf.* **1977**, *2*, 565-573.
114. Fenn, M. E.; Coffey, M. D. *Phytopathology* **1984**, *74*, 606-611.

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch008

115.Whiley, A. W.; Pegg, K. G.; Saranah, J. B.; Forsberg, L. I. *Aust. J. Exp. Agr.* **1986**, *26*, 249-253.

116.Fenn, M. E.; Cogffey, M. D. *Phytopathology* **1989**, *79*, 76-82.

117.Griffith, J. M.; Smillie; Grant, B. R. *J. Gen. Microbiol.* **1990**, *136*, 1285-1291.

118.Niere, J. O.; De Angelis G.; Grant, B. R. *Microbiol.* **1994**, *40*, 1661-1670.

119.Cassaigne, A.; Lacoste, A.-M.; Neuzil, E. *Biochim. Biophys. Acta* **1971**, *252*, 506-515.

120.McMullan, G.; Harrington, F.; Quinn, J. P. *Appl. Environ. Microbiol.* **1992**, *58*, 1354-1360.

121.McMullan, G.; Quinn, J. P. *Biochem. Biophys. Res. Commun.* **1992**, *184*, 1022-1027.

122.McMullan, G.; Quinn, J. P. *J. Bacteriol.* **1994**, *176*, 320-324.

123.McGrath, J. W.; Wisdom, G. B.; McMullan, G.; Larkin, M. K.; Quinn, J. P. *Eur. J. Chem.* **1995**, *234*, 225-230.

124.Kulakova, A. M.; Kulakov, L. A.; Quinn, J. P. *Gene* **1997**, *195*, 49-53.

125.Burns, B. P.; Mendz, G L.; Hazell, S. L. *J. Bacteriol.* **1998**, *180*, 5574-5579.

126.Collins, K. D.; Stark, G. R. *J. Biol. Chem.* **1971**, *246*, 6599-6605.

127.Johnson, L. M.; Talbot, H. W. *Experientia* **1983**, *39*, 702-706.

128.Yoshida, T.; Sethunathan, N. *Can. J. Microbiol.* **1973**, *19*, 873-875.

129.Daughton, C. G.; Hsieh, D. P. *Appl. Environ. Microbiol.* **1977**, *34*, 175-184.

130.Siddaramappa, R.; Rajaram, K. P.; Sethunathan, N. *Appl. Microbiol.* **1973**, *26*, 846-849.

131.Bourquin, A. W. *Appl. Environ. Microbiol.* **1977**, *33*, 356-362.

132.Rosenberg, A.; Alexander, M. *Appl. Environ. Microbiol.* **1979**, *37*, 886-891.

133.Munnecke, D. M.; Hsieh, D. P. H. *Appl. Environ. Microbiol.* **1976**, *31*, 63-69.

134.De Matteis F. In *Sulphur Containing Drugs and Related Organic Compounds*; Damani, L. A. Ed.; Ellis Harwood, Chichester 1989, pp. 9-34.

135.Munnecke, D. M. *Appl. Environ. Microbiol.* **1976**, *32*, 7-13.

136.Mulbry, W. W.; Karns, J. S. *Appl. Environ. Microbiol.* **1989**, *55*, 289-293.

137.McDaniel, C. S.; Harper L. L.; Wild, J. R. *J. Bacteriol.* **1988**, 170, 2306-2311.

138.Mulbry, W. W.; Karns, J. S. *J. Bacteriol.* **1989**, *171*, 6740-6746.

139.Donarski, W. J.; Dumas, D. P.; Heitmeyer, D. P.; Lewis, V. E.; Raushel, F. M. *Biochemistry* **1989**, *28*, 4650-4655.

140.Dave, K. I.; Miller, C. E.; Wild, J. R. *Chem.-Biol. Interactions* **1993**, *87*, 55-68.

141.Harper, L. L.; McDaniel, C. S.; Miller; C. E.; Wild, J. R. *Appl. Environ. Microbiol.* **1988**, *54*, 2584-2589.

142. Serdar, C. M.; Rhode, M. F.; Murdock, D. C. *Bio/Technology* **1989**, *7*, 1151-1155.

143.Wolfenden, R.; Spence G. *Biochim. Biophys. Acta* **1967**, *146*, 296-298.

144.Dumas, D. P.; Durst, H. D.; Landis, W. G.; Raushel F. M. *Arch. Biochem. Biophys.* 1990, 277, 155-159.

145.Cheng, T.-C.; Calomiris, J. *J. Enzyme Micro-Technol.* **1996**, *18*, 597-601.

146.Cheng, T.-C.; Harvey, S. P.; Chen, G. L. *Appl. Environ. Microbiol.* **1996**, *62*, 1636-1641.

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch008

147.Cheng, T.-C.; Liu, L.; Wang, B.; Wu, J.; DeFrank, J. J.; Anderson, D. M.; Rastogi, V. K.; Hamilton, A. B. J. Ind. Microbiol. 1996, 17, 554-563.

148.DeFrank, J. J.; Cheng, T.-C. *J. Bacteriol.* **1991**, *173*, 1938-1943.

149.Amitai G.; Adani, R.; Sod-Moriah, G.; Rabinowitz, I.; Vincze, A.; Leader, H.; Chefetz, B.; Libovitz-Persky, L.; Friesem, D.; Hadar, Y. *FEBS Lett.* **1998**, *438*, 195-200.

150.Zhang, Y.; Autenrieth, R. L.; Bonner, J. S.; Harvey, S. P.; Wild, J. S. *Biotechnol. Bioeng.* **1999**, *64*, 221-231.

151.Mulbry, W.:, Rainina, E. *ASM News* **1998**, *64*, 325-331.

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch008

Chapter 9

Plant and Fungal Cytochrome P-450s: Their Role in Pesticide Transformation

C. P. Mougin[1], M.-F. Corio-Costet[2], and D. Werck-Reichhart[3]

[1]INRA, Unité de Phytopharmacie et Médiateurs Chimiques, Route de Saint-Cyr, F-78026 Versailles Cedex, France
[2]INRA, Unité de Recherches en Santé Végétale, Domaine de la Grande-Ferrade, BP 81, F-33883 Villenave d'Ornon Cedex, France
[3]CNRS, Département d'Enzymologic Moléculaire et Cellulaire, Institut de Biologie Moléculaire des Plantes, UPR 406, 28 Rue Goethe F-67083 Strasbourg Cedex, France

Downloaded by OHIO STATE UNIV LIBRARIES on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch009

Cytochromes P-450 (P-450s) constitute a large family of oxygenases catalyzing phase I metabolism of various xenobiotics, including pesticides. In most cases, they mediate reactions resulting in detoxification of the parent molecule. Plant P-450s play an important role in herbicide metabolism, by constituting a key factor of herbicide selectivity, and of herbicide resistance recently observed in some weed biotypes. Definitive proof of P-450-mediated pesticide metabolism in filamentous fungi has not yet been obtained. However, indirect evidence supports such an hypothesis. The detoxification and degradation potential of P-450s from plants or microorganisms could be exploited for bioremediation of water, industrial waste, and soil contaminated with pesticides. A more comprehensive knowledge of these enzymes, including functional characterization of genes and proteins, remains necessary for an efficient management of P-450 potentialities.

In Pesticide Biotransformation in Plants and Microorganisms; Hall, J., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2000.

Downloaded by OHIO STATE UNIV LIBRARIES on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch009

Living organisms are all directly or indirectly exposed to foreign organic molecules (or xenobiotics), called pesticides. In most cases, xenobiotics are absorbed by living cells and undergo biochemical modifications. These modifications may result from several types of phase I reactions, such as hydrolysis, reduction, or oxidation, and from various conjugation reactions. Such reactions participate to different extents in the metabolism of each pesticide. A survey shows that, among the initial steps responsible for the transformation of parent molecules into primary metabolites, the most frequent are oxygenation reactions. In animals, it has been known for many years that such reactions are mainly mediated by P-450s. In plants, evidence for the role of P-450 in the oxygenation of a pesticide, *i.e.* the *N*-demethylation of the herbicide monuron [*N'*-(4-chlorophenyl)-*N,N*-dimethylurea], was obtained at the end of the 1960's (*1*). In the case of filamentous fungi, no data are yet available concerning pesticides.

The purpose of this chapter is to present the main structural features and functions of P-450s, and to exemplify their involvement in the metabolism of pesticides in higher plants and filamentous fungi. As far as possible, data are found in recent reviews listed in the literature section, and are concerned with enzyme structure and phylogeny (*2,3*), reactions and functions (*4,5,6*), nomenclature (*7*), plant P-450 molecular enzymology (*8,9*), and microbial and fungal P-450s (*10,11,12,13*).

Properties and Functions of Plant and Microbial P-450s: Similarities and Divergences

Properties

P-450s constitute a large family of heme-thiolate proteins, widely distributed among living organisms. In most cases, they function as monooxygenases, by binding and activating molecular oxygen, incorporating one of its atoms into an organic substrate, and reducing the second atom to form water, according to the following reaction:

$$RH + O_2 + NAD(P)H + H^+ \text{-->} ROH + H_2O + NA(D)P^+$$

The result of catalysis, depending on the P-450 protein and its substrate, is in most cases hydroxylation, but epoxidation, heteroatom dealkylation, deamination, isomerization, C-C or C=N cleavage, dimerization, ring formation, dehydration, dehydrogenation or reduction have also been reported.

For most eucaryotic P-450s, a FAD/FMN-dependent NADPH-P-450 reductase is needed to transfer the electrons used for oxygen activation from cytosolic NADPH. In plants and filamentous fungi, as well as in other eucaryotic organisms, P-450s and reductases are usually microsomal membrane-bound proteins, exposed to the cytosol. Nevertheless, soluble forms of P-450s, coupling P-450 and reductase in a single fusion protein, have also been found in bacteria and fungi (*12*). A second type of P-450s has been identified in prokaryotes and animal mitochondria. In addition to a flavoprotein, it needs a small redox iron-sulfur protein (ferredoxin) to transfer electrons from NAD(P)H to the terminal P-450 component. Such P-450s are usually soluble or associated with inner mitochondrial membranes. A third class of P-450s does not require an auxillary redox partner or molecular oxygen to catalyze the rearrangement of hydroperoxides. Some of such plant P-450s seem to be located in the plastids (*14*).

When reduced, P-450s can bind carbon monoxide instead of oxygen, forming a complex that shifts the Soret absorption maximum between 447 and 452 nm. As a result, the enzymatic reaction is blocked, but inhibition can be partly reversed by light, with a maximum of efficiency around 450 nm. A second species absorbing at 420 nm, that is considered to be an inactive form of P-450, is often simultaneously detected. A shift of the Soret of the oxidized P-450s can also be detected. When a substrate binds the active site, it results in a so-called type I spectrum with a peak near 390 nm and a trough around 420 nm. After addition of inhibitors such as heterocyclic compounds, it results in a type II absorbance change (peak around 430 nm, and trough at 390-400 nm). Such absorption changes are indicative of shifts in the spin equilibrium and redox potential of the cytochrome.

P-450s are encoded by a superfamily of genes. The sequences of more than 500 of them have already been recorded in all living organisms (*7*). They are named and grouped into more than 150 families, according to the amino acid sequences of the deduced proteins. With a few exceptions, based on phylogenetic considerations, protein sequences within a given gene family are >40% identical. When two sequences are more than 55% identical, proteins are designated as members of the same subfamily. Following the prefix CYP, numbers designate families, and letters subfamilies. Plant P-450s correspond to the families CYP71 to CYP99, then CYP701 and above. Fungal P-450s comprise families CYP51 to CYP66.

The first plant P-450 gene, CYP71A1, was cloned at the beginning of the 1990's. There was then an explosion in the rate at which genes encoding plant P-450s have been identified. More than 200 genes have been registered in plants, 40 in fungi (including yeast), and 90 in prokaryotes. The apoprotein sequences (45 to 60 kDa) are highly variable, but their three-dimensional structures seem to be somewhat similar. The most highly conserved part of all P-450s is the core surrounding the heme prosthetic group. The proximal side of the heme is formed of a loop including the cysteine residue (serving as the sixth ligand of the iron) and the eleven most highly conserved residues of the protein. Other conserved elements, found on the distal side, are responsible for oxygen binding and activation, and for the transfer of the protons to the activated oxygen. Most P-450s seem to be anchored to membranes primarily via a 30 to 50 amino acids *N*-terminal segment. Information on targeting, insertion and topography of fungal, and especially plant P-450s remains sparse.

Knowledge concerning the regulation of plant and fungal P-450s is only starting to accumulate. In plants, expression generally seems to follow a developmentally regulated tissue-specific pattern. Physicochemical (light, osmotic stress, wounding), physiological (infection, ageing, hormones) factors, or xenobiotics (agrochemicals, ethanol, or drugs like phenobarbital or aminopyrine) were reported to induce plant P-450s. In many cases, expression is very low or undetectable in the absence of

Downloaded by OHIO STATE UNIV LIBRARIES on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch009

induction. Plant or fungal P-450 enzymes can be inhibited by mechanism-based inactivators (heme or apoprotein alkylating agents, like 1-aminobenzotriazole (ABT) or acetylenic substrate analogues), heterocyclic molecules (imidazole, pyrimidine, triazole derivatives) or methylenedioxy compounds (*e.g.*, piperonyl butoxide (PBO) or piperonylic acid). Some of these compounds inhibit a broad range of enzymes, while others seem to be more selective. Selective induction and inhibition can be used to differentiate between P-450s involved in specific oxygenation reactions.

Plant and fungal P-450 systems so far remain less understood than their mammalian homologues, because these proteins are typically present in low abundance and are often unstable during purification. In general, P-450 amounts in non-induced plants, fungi and bacteria are less than 0.1 nmole mg^{-1} microsomal proteins, and represent only the one-tenth the amount naturally expressed in mammalian liver.

Downloaded by OHIO STATE UNIV LIBRARIES on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch009

Functions

Most of the plant P-450 genes recently isolated have not been attributed with a function, either *in vitro* or *in vivo*. The natural substrates reported so far are plant specific secondary metabolites, like phenylpropanoids, isoprenoids, alkaloids, plant growth regulators, amino acid derivatives, or natural compounds also present in fungi and animals such as sterols or fatty acids. Some plant P-450s are also capable, probably fortuitously, of metabolizing xenobiotics. Data accumulated in the last ten years, indicate that they constitute the major oxidative pathway involved in herbicide metabolism. In fungi, the involvement of P-450 systems in many complex bioconversions processes (metabolism of alkanes, synthesis of sterols and antibiotics, reduction of nitrogen oxide) has been demonstrated in recent years. Among xenobiotics, only polycyclic aromatic hydrocarbon (benzo[a]pyrene) have been identified as substrates of fungal P-450s. Bacterial P-450s, metabolizing herbicides such as sulfonylureas, carbamates or triazines, have also been reported.

Role of Plant P-450s in Herbicide Metabolism and Selectivity

Metabolic detoxification is one of the main mechanisms of herbicide selectivity, and is responsible in many cases for the tolerance of major crops and resistant weeds. It is now well established that P-450-catalyzed reactions are often at the origin of this phenomenon (*15,16,17*). Evidence indicates qualitative and quantitative differences in P-450 contents from plant to plant. For that reason, species and cultivars exhibit differences in herbicide metabolism, as well as differential enzymatic induction or inhibition.

Phenylureas provide the best-documented example of herbicide metabolism in higher plants. Monuron dealkylation in cotton (*Gossypium hirsutum*) was one of the first P-450-dependent reactions to be characterized as early as 1969 (*1*). Different reactions associated with P-450-dependent chlortoluron [*N'*-(3-chloro-4-methylphenyl)-*N,N*-dimethylurea] metabolism were later reported in many plants, including both crops and weeds. In addition, the only genes of herbicide-metabolizing plant P-450s characterized so far, are all involved in phenylurea detoxification.

Metabolism of Chlortoluron in Plants

Chlorotoluron is active against a number of monocots and dicots, and is principally detoxified by hydroxylation of the ring-methyl (CPUH), or *N*-demethylation (CPUDM). The ring-methyl hydroxylated and the di-*N*-demethylated products are non-phytotoxic. The polar metabolites can be partly conjugated to glucose, resulting in detoxification.

Chlortoluron provides a very good example of metabolism associated with herbicide selectivity between weeds and crops. In the tolerant winter wheat (*Triticum aestivum*), the half-life of chlortoluron is less than 24 hours. The main metabolite is the non-phytotoxic *ring*-methyl hydroxylated derivative. In the susceptible weed blackgrass (*Alopecurus myosuroides)*, the main metabolite is the mono-*N*-demethylated compound. Phytotoxicity of this metabolite has a half-life greater than 24 hours. In the tolerant weed Persian speedwell (*Veronica persica)*, on the other hand, the herbicide has a half-life of only 6 hours, and is converted to the non-phytotoxic di-*N*-demethylated product.

Identification of the enzymes responsible for the oxidation reactions in wheat was first attempted using *in vivo* experiments. The first evidence was reported by Gaillardon *et al.*, who showed that administration of PBO and ABT increased the toxicity of chlortoluron (*18*). ABT strongly inhibited both CPUH and CPUDM activities (*19*). This finding was later extended to other tolerant species, but *N*-demethylation of chlortoluron was little affected by ABT in Persian speedwell (*20)*.

Metabolism of Chlortoluron in Cell Cultures

Similar experiments were performed with cell cultures. CPUDM was more active in wheat cells than in the whole plant. ABT, and the plant growth retardants, tetcyclacis [5-(4-chlorophenyl)-3,4,5,9,10-pentaaza-teracyclo-5,4,1,$0^{2,6}$,$0^{8,11}$-dodeca-3,9-diene] and paclobutrazol [(2*RS*,3*RS*)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1*H*-1,2,4-triazol-1-yl)pentan-3-ol], inhibited herbicide transformation (*21*). By contrast, metabolism was increased after pretreatment of cells with 2,4-D [(2,4-dichlorophenoxy)acetic acid], the herbicide safener cyometrinil, {(Z)-α[(cyanomethoxy)-imino]benzene acetonitrile} or the imidazole fungicide prochloraz {*N*-propyl-*N*-[2-(2,4,6-trichlorophenoxy)ethyl]imidazole-1-carboxamide} (*21*). 2,4-D and cyometrinil also stimulated chlortoluron metabolism in Persian speedwell cells, by inducing expression of a new CPUH activity (F. Cabanne, unpublished results).

Downloaded by OHIO STATE UNIV LIBRARIES on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch009

Metabolism of Chlortoluron in Microsomal Preparations

All *in vivo* results strongly suggested that oxidative transformation of chlortoluron could be mediated by P-450s. A first proof *in vitro* was obtained for CPUDM activity with microsomes isolated from Jerusalem artichoke tubers (*Helianthus tuberosus*) (*22*). Definitive proof for CPUH activity arose from assays with microsomal preparations isolated from wheat (*23*). Metabolism of chlortoluron was also detected in microsomes from Persian speedwell (N. Polge, unpublished results). A higher efficiency of tuber slices and cell cultures versus etiolated seedlings was due to the fact that they could be pretreated with inducers prior to the preparation of microsomes (*22,24*). In all species, both CPUH and CPUDM exhibited properties of P-450-mediated activities. In wheat and Jerusalem artichoke, spectral evidence also confirmed P-450 involvement in chlortoluron metabolism. Reduced carbon monoxide spectra showed that microsomes isolated from wheat cells grown for 48 h in the presence of 300 µM 2,4-D, contained about 250 pmol P-450 per mg microsomal protein (Figure 1A). A type I spectrum was obtained by incubating such microsomes with chlortoluron, indicating binding of the herbicide to the catalytic site of P-450 (Figure 1B). In addition, the fungicide procloraz, inhibiting chlortoluron oxidation in wheat microsomes, induced the formation of type II spectra when applied at 100 to 400 µM, which suggested its binding to P-450 heme iron (Figure 1C). Microsomal preparations from wheat were also able to *N*-demethylate the phenylurea herbicides isoproturon [*N*'-(4-isopropylphenyl)-*N*,*N*-dimethyl-urea] and diuron [*N*'-(3,4-dichlorophenyl)-*N*,*N*-dimethyl-urea] (*25*).

In wheat, P-450 effectors differently affected CPUH and CPUDM. First, inhibitors such as heterocyclic molecules and methylenedioxy compounds did not inhibit the activities to the same extent. Only CPUH was very sensitive to ABT. On the other hand, CPUDM was more strongly stimulated than CPUH when cells were treated with P-450 inducers. Finally, only CPUDM was active with organic hydroperoxides as electron donors. Thus, at least two P-450s are probably involved in the metabolism of chlortoluron in wheat. Similar conclusions were drawn concerning CPUH and CPUDM activities from Persian speedwell.

Interspecific differences were also evidenced by different sensitivities towards carbon monoxide, tetcyclacis and ABT (Table I). In both wheat and Persian speedwell, enzymatic systems responsible for chlortoluron metabolism exhibited an induction pattern similar to laurate hydroxylase, used as physiological P-450 marker. In wheat, the activities were, however, clearly distinguished by using specific inhibitors (*26*).

Following the pioneering work performed with phenylureas, evidence for the involvement of P-450s in the metabolism of pesticides belonging to other major classes of herbicides {sulfonylureas, alloxy-phenoxyalkanonates, chloroacetanilides, imidazolinones, bentazon [3-(1-methylethyl)-(1*H*)-2,1,3-benzothiadiazin-4(3*H*)-one 2,2-dioxide], flumetsulam [*N*-(2,6-difluorophenyl)-5-methyl[1,2,4]triazolo[1,5-a]pyrimidine-2-sulfonamide]} have been obtained in major crops [wheat, corn (*Zea mays*), barley (*Hordeum vulgare*), sorghum (*Sorghum bicolor*)], or weeds.

Downloaded by OHIO STATE UNIV LIBRARIES on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch009

Downloaded by OHIO STATE UNIV LIBRARIES on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch009

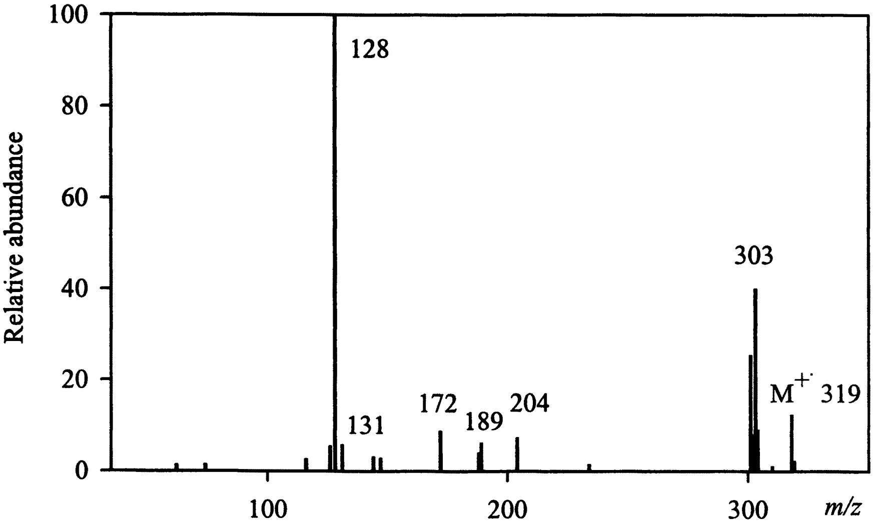


Figure 1. Difference spectra of wheat cell microsomes. A, reduced carbon monoxide spectrum; B, chlorotoluron-enzyme type I binding spectrum (herbicide concentrations were 0, 100 and 160 μM); C, procloraz-enzyme type II binding spectrum (final concentrations of fungicide ranged from 100 to 400 μM). (Reproduced from reference 25. Copyright 1992 Elsevier).

Table I. Inhibition of Chlorotoluron Metabolism by P-450 Inhibitors in Wheat and Persian Speedwell

Inhibitors		% inhibition			
		Wheat		*Speedwell*	
		CPUH	CPUDM	CPUH	CPUDM
Carbon monoxide		50	35	75	98
Tetcyclacis	10 µM	20	10	74	45
	100 µM	60	40	99	80
ABT	10 µM	74	57	0	0
	100 µM	100	100	35	7

P-450 Genes Involved in Phenylurea Metabolism

Paradoxically, the first gene shown to encode a P-450 protein metabolizing phenylureas, and to increase plant tolerance to chlortoluron was the benzo[a]pyrene activating CYP1A1 from rat liver (*27*). This P-450 displayed both CPUH and CPUDM activities, but their turnovers were quite low. Recently, the genes of two other phenylurea metabolizing P-450s were isolated from higher plants (*28,29*). The gene of the *H. tuberosus* P-450 previously reported to catalyze chlortoluron *N*-demethylation, was isolated on the basis of its inducibility by aminopyrine (*28*). CYP76B1 expression in yeast confirmed that it had chlortoluron di-*N*-demethylase, but no CPUH activity. CPUDM catalytic efficiency was surprisingly high and similar to that observed with physiological substrates. CYP76B1 also catalyzed the *O*-dealkylation of various 7-alkoxy-coumarins and 7-alkoxyphenoxazones, like CYP1A1, but had no benzo[a]pyrene oxygenase activity. Another gene, CYP71A10 was isolated from soybean (*29*). CYP71A10 has properties more similar to CYP1A1 with regard to phenylurea metabolism, and catalyzes both CPUH and CPUDM activities with turnover rates much lower than that of CYP76B1. Both P-450s metabolize a broad range of phenylureas and are capable of increasing phenylurea tolerance of susceptible plants.

Role of Plant P-450s in Herbicide Cross-Resistance in Weeds

As exemplified by the case of chlortoluron, the level of tolerance conferred by P-450-mediated metabolism of herbicides can be quite high in crops, but also in weeds. Herbicide resistance in weeds is in general more frequently related to mutation of the target site than to enhanced metabolism. Nevertheless, cases of weed resistance resulting from increased P-450 metabolism following intensive use of herbicides have

Downloaded by OHIO STATE UNIV LIBRARIES on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch009

been reported in Australian biotypes of rigid ryegrass (*Lolium rigidum*) (*30*) and European biotypes of blackgrass (*31,32*) in the ten last years. The nature of the metabolites formed, and the effects of known P-450 inhibitors, gave strong evidence of the involvement of P-450 in the resistance of these weeds. Despite intensive efforts, definitive proof could never be established *in vitro* using microsomal preparations of rigid ryegrass, although direct evidence was obtained in blackgrass.

The development of increased metabolism sometimes results in cross-resistance to several herbicides from different classes and leads to multiple resistant weed populations (*30*). Cross-resistance may also result from the cross-pollination of plants with resistant target sites, and plants with increased metabolism. The mechanism of acquisition of metabolism-dependent herbicide resistance in weeds is not yet understood.

Downloaded by OHIO STATE UNIV LIBRARIES on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch009

Role of Plant P-450s in Fungicide Metabolism in the Host Plant

Fenpropimorph [(±)-*cis*-4-[3-(4-*t*-butylphenyl)-2-methylpropyl]-2,6-dimethyl morpholine] is a derivative acting as a sterol biosynthesis inhibitor (SBI), that has been widely used during recent decades as a systemic fungicide against powdery mildew and rust diseases of cereals. This fungitoxic molecule, however, was also found to interfere with the biosynthesis of the sterols of the host plant by replacing Δ^5-sterols by Δ^8-sterols and cyclopropylsterols *(33)*. This accumulation of abnormal sterols is due to the inhibition of two target enzymes: the cycloeucalenol obtusifoliol isomerase (COI) and Δ^8->Δ^7-sterol-isomerase. This section reports new experimental results that concerned interactions of fenpropimorph and agrochemicals with wheat plants.

Experimental

To study the effects of agrochemicals on sterol profiles, wheat seedlings were germinated in vermiculite in the dark at 20°C, and then watered daily with solutions containing fenpropimorph, ABT or naphthalic anhydride [naphthalene-1,8-dicarboxylic anhydride] (each at 2 mg L^{-1}) over 10 days. Then, shoots and roots were measured. Sterols were extracted and analyzed as reported (*34*). To evaluate the effects of agrochemicals on wheat P-450 content, seeds were soaked and germinated on paper immersed in water containing the chemicals, for 5 days in the dark at 25°C. Approximately 40 g of etiolated seedlings were harvested and used for the preparation of microsomes (*24*). Cytochromes were measured according to Omura and Sato (*35*). Fenpropimorph [ring-^{14}C] was incubated with microsomes using published protocols (*24*), prior to extraction for TLC and GC-MS analysis.

Combined Effects of Fenpropimorph and Agrochemicals on Sterol Profiles

With respect to untreated controls, seedlings treated with fenpropimorph accumulated abnormal sterols (Δ^8-sterols and cyclopropylsterols) in leaves and roots, whereas the Δ^5-sterols (initially present) declined to very low levels (Table II). The modification of the sterol profiles was correlated with an increase in abnormal sterols from less than 10% to 85.7% in leaves and 95.9% in roots. ABT and naphthalic anhydride alone increased the amounts of Δ^8-sterols to 30%, by reducing the levels of Δ^5-sterols (not shown).

A higher amount of cyclopropylsterols accumulated in roots of wheat seedlings treated with a mixture of fenpropimorph and ABT (87.9 %), compared to seedlings. treated with fenpropimorph alone.reated (58 %). ABT activity led to a synergistic effect on fenpropimorph action in roots. As the first target of fenpropimorph on sterol biosynthesis enzymes is the COI, the synergistic effect of ABT may result in a complete inhibition of this enzyme.

The combination of fenpropimorph and naphthalic anhydride led to a significant change in sterol biosynthesis mainly in leaves (Table II). Total amounts of Δ^8-sterols and cyclopropylsterols decreased from 85.7% in leaves treated with fenpropimorph alone, to 60.2% in presence of naphthalic anhydride. On the other hand, we observed an increase in the amount of total sterols from 14.3 to 39.8% in leaves and from 4.1 to 10.9% in roots. This suggests that naphthalic anhydride, an inducer of plant P-450s, may either activate fenpropimorph detoxification, or stimulate a P-450 enzyme involved in sterol biosynthesis, such as the 14α-demethylase.

Downloaded by OHIO STATE UNIV LIBRARIES on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch009

Table II: Sterol Distribution in Wheat Seedlings after 10-Day Treatments with Fenpropimorph (Fen), or Fenpropimorph + ABT, Fenpropimorph + Naphthalic Anhydride (NA), in Leaves (L) and Roots (R)

Sterol distribution	*Control*		*Fen.*		*Fen. + ABT*		*Fen. + NA*	
(% of total sterol)	L	R	L	R	L	R	L	R
Δ^5 Sterols	94.0	91.3	13.3	2.8	10.5	7.0	37.5	8.5
Δ^7 Sterols	1.7	1.4	1.0	1.3	1.0	0.4	3.2	2.4
Δ^8 Sterols	1.7	1.9	46.8	31.3	40.9	5.0	29.2	25.1
Cyclopropylsterols	2.4	4.4	38.9	58	47.5	87.9	31.0	64.0
Normal sterols	95.7	92.7	14.3	4.1	11.5	7.4	39.8	10.9
Abnormal sterols	4.3	7.3	85.7	95.9	88.5	92.5	60.2	89.1

Effects of Fenpropimorph with Agrochemicals on Wheat P-450 Content

Seedlings treated with fenpropimorph alone (0.05% by weight of seeds) showed a 1.7-fold increase in microsomal P-450 content from 113 to 196 pmoles mg^{-1} protein

(Table III). When fenpropimorph was combined with naphthalic anhydride or clofibrate, the P-450 level increased to 300 pmoles mg^{-1}. In combination with fenpropimorph, PBO, ABT and tetcyclacis slightly decreased P-450 contents to 130-160 pmoles mg^{-1}, but high amounts of P-420 were detected. Inducing effects may be blocked by the last three compounds, which can bind to or inactivate the neo-formed enzymes (*36*). We thus may not detect any strong inducing effect.

Table III : Effect of Treatment of Seedlings with Agrochemicals On Microsomal P-450 Content

Treatment	*Amount** (%)	*P-450*** (pmoles mg^{-1})
Control (water)	0	113
Fenpropimorph	0.05	196
Fen. + PBO	0.05+ 0.05	160
Fen. + naphthalic an.	0.05 + 0.1	302
Fen. + ABT	0.05 + 0.05	130
Fen. + tetcyclacis	0.05 + 0.025	138
Fen. + clofibrate	0.05 + 0.05	340

NOTE: * by weight of seeds, ** SD does not exceed 12% of value.

Downloaded by OHIO STATE UNIV LIBRARIES on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch009

Involvement of P-450 in Fenpropimorph Metabolism

Fenpropimorph was metabolized to an oxygenated compound by microsomal fractions isolated from wheat seedlings. The mass spectrum of the metabolite fitted with an oxidized derivative either being hydroxylated on the *t*-butyl group or *N*-oxidized (Figure 2). The reaction required NADPH as cofactor, and was partly inhibited by carbon monoxide. The enzymatic activity was low (93 pmoles mg^{-1} h^{-1}), but increased 2-fold (180 pmoles mg^{-1} h^{-1}) when seedlings were pretreated with naphthalic anhydride, whether or not the fungicide was present. Thus, the fungicide oxidation was assumed to be P-450-mediated in wheat.

These results confirm that agrochemicals acting as P-450 effectors can modify the metabolism of a pesticide, and may lead to toxic effects on the host plant. These experiments also suggest that these agrochemicals can modulate the overall metabolism of a plant, by increasing or inhibiting physiological activities, thereby affecting plant growth.

Downloaded by OHIO STATE UNIV LIBRARIES on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch009

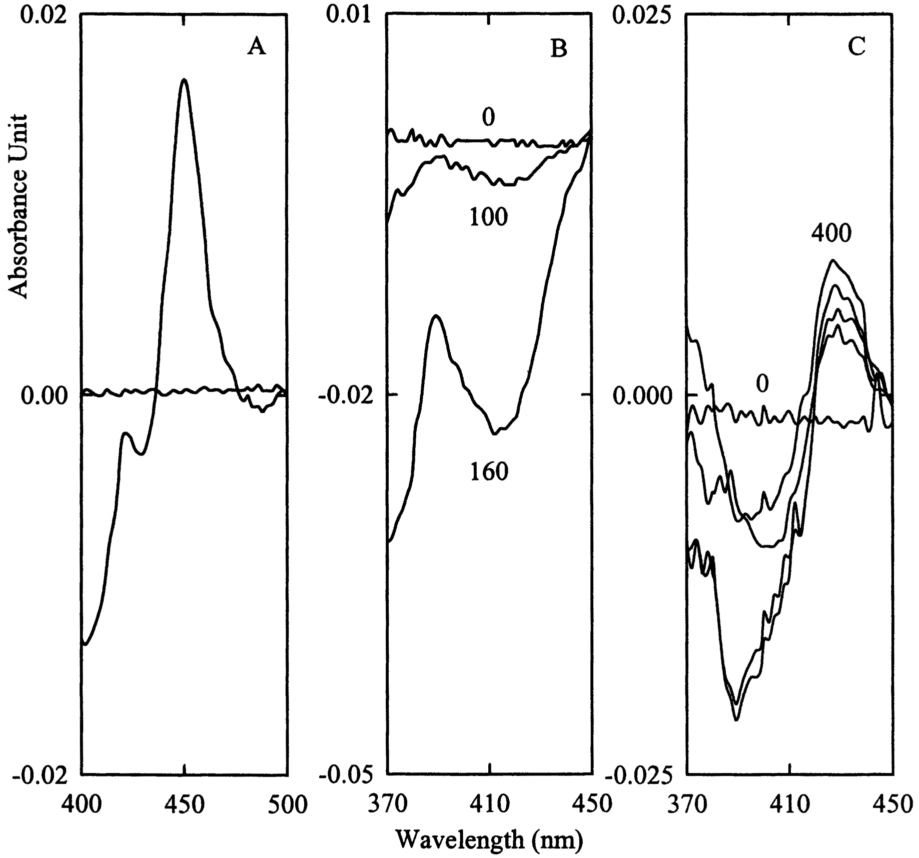


Figure 2. GC-EI-MS spectrum of the oxygenated metabolite formed from fenpropimorph by wheat microsomal preparations.

Fungal P-450s: Tools for Bioremediation

Remediation of water and soils contaminated with organic pollutants is a becoming a major challenge because of widespread pollution and its detrimental implication in human health. As a general rule, removing the contaminants (decontamination) is preferred to sequestering them (stabilization). Many organisms, including plants and microorganisms, can be used for that purpose. We have studied filamentous fungi as tools for the bioremediation of soils contaminated with pesticides or polycyclic aromatic hydrocarbons. The major advantage of such organisms is their direct contact with the pollutants in the solid, liquid and vapor phase of the soil, avoiding problems of uptake and translocation occurring with plants. Moreover, fungi can transform a wide range of pollutants, with a good resistance to possible toxic effects of the biocides. Fungi usually release most of their metabolites, inducing a synergism with closely associated indigenous microflora.

Downloaded by OHIO STATE UNIV LIBRARIES on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch009

Evidence of P-450s in Filamentous Fungi

Our model system is the ligninolytic strain, *Phanerochaete chrysosporium*. This basidiomycete has been studied for many years for its production of extracellular oxidases, namely lignin and manganese-dependent peroxidases. Recently, we have confirmed the presence of intracellular enzymes in the fungus, grown in liquid cultures. A reduced P-450-CO spectrum was obtained using *P. chrysosporium* microsomal preparations. Unfortunately, the amount of P-450 was low (89 pmol mg^{-1} microsomal proteins), and we failed to detect xenobiotic metabolism in this preparation *(37)*. This could be attributed to the high level of P-420, which is indicative of partial degradation of the enzyme during isolation.

Involvement of P-450s in Lindane Transformation

P. chrysosporium metabolized the insecticide lindane [1α,2α,3β,4α,5α,6β-hexachlorocyclohexane] to polar compounds (*i.e.,* tetrachlorocyclohexene epoxide and tetracyclohexenol) and carbon dioxide (*38*). The same metabolites are formed by microsomal fractions from rat liver. ABT treatment of *P. chrysosporium* cultures drastically reduced lindane metabolism, inhibiting the formation of all metabolites as well as carbon dioxide. On the other hand, phenobarbital treatment did not modify lindane mineralization, but increased the oxidation of tetrachlorocyclohexene to tetrachlorocyclohexene epoxide and tetracyclohexenol. These indirect effects are consistent with the involvement of P-450s in one or more steps of lindane metabolism.

Soil Bioremediation

Biotransformation of lindane was investigated in sterilized and non-sterilized soils, with or without *P. chrysosporium* (*39*). After spore inoculation of the sterilized soil, the fungal biomass increased rapidly during the first week, but was less rapid during an additional 8 weeks. Only limited fungal development occurred in non-sterile soils. However, lindane mineralization, by indigenous microbial activity, was increased 2-fold by inoculation with *P. chrysosporium* (Table IV). Extractable amounts of residual lindane were similar in soils, with or without inoculation. Thus, *P. chrysosporium* modified lindane degradation by increasing mineralization to carbon dioxide, or its conversion to volatile metabolites by the indigenous microflora.

Table IV. Mass-Balance Analysis of ^{14}C in Soil Cultures after a 9-Week Incubation

Treatment	*Fraction* (% initial radioactivity)				
	CO_2	Extractable	Non-extractable	Volatile	Total
Sterile, non inoculated	0.0	77.7	5.2	n.d.	82.9
Sterile, inoculated	0.7	74.3	6.4	n.d.	81.4
Non-sterile, non inoculated	21.6	9.7	18.4	2.6	52.4
Non-sterile, inoculated	49.1	9.1	14.4	n.d.	72.6

NOTE: n.d.: not detected

SOURCE: Reproduced from reference 39. Copyright 1997 Elsevier.

These results confirm that the bioremediation of contaminated soils may be achieved using a bioaugmentation approach with filamentous fungi in a synergistic relationship with the indigenous microbial community. High bioremediation efficiency is only expected when pollutants exhibit sufficient water solubility and bioavailability.

Some filamentous fungi are able to degrade an extremely diverse range of persistent or toxic environmental pollutants. This ability is often due to the production of several families of extracellular enzymes, such as peroxidases or laccases. The extracellular systems (well-known in white-rot fungi) use non-specific mechanisms, and are regulated by nutrients. Consequently, they can degrade chemicals present at levels below the concentrations required for effective enzyme induction.

Downloaded by OHIO STATE UNIV LIBRARIES on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch009

Concluding Remarks

P-450s are clearly involved in pesticide metabolism in plants and fungi, but the genes encoding P-450 proteins responsible for this metabolism are only at the initial stage of characterization. It is already clear that genes with redundant functions will be found. The isolation and functional characterization of more genes is needed in order to determine their relative importance for herbicide metabolism and selectivity in plants, and their potential for bioremediation. So far, there are only indirect indications that some plant P-450s may metabolize a broad range of herbicides. To date only one recent report confirms this with an isolated recombinant enzyme (*40*). Isolation of herbicide metabolizing P-450 genes is one of the keys for understanding the mechanism(s) of weed cross-resistance, and regulation of herbicide metabolism.

Direct or random genetic mutagenesis of plant or fungal P-450 genes may also enhance our understanding of these enzymes and their role in specific pesticide or pollutant metabolism. These genes can also be engineered for improved catalytic activity. Over-expression of native and modified genes in plants or microorganisms, together with suitable P-450 reductases, should be useful for increasing resistance of susceptible crops, or for organic pollutant removal. Manipulation of P-450 enzymes is thus a promising long-term challenge.

Downloaded by OHIO STATE UNIV LIBRARIES on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch009

Literature Cited

1. Frear, D. S.; Swanson, H. R.; Tanaka, F. S. *Phytochemistry* **1969**, *8*, 2157-2169.
2. Hasemann, C. A.; Kurumbail, R. G., Boddupalli, S. S.; Peterson, J. A.; Deisenhofer, J. *J. Current Biol.* **1995**, *2*, 41-62.
3. Durst, F.; Nelson D. R. *Drug Metab. Drug Interact.* **1995**, *12*, 189-206.
4. Halkier, B. A. *Phytochemistry* **1996**, *43*, 1-21.
5. Mansuy, D. *Comp. Biochem. Physiol. C.* **1998**, *121*, 5-14.
6. Schuler, M. A. *Crit. Rev. Plant. Sci.* **1996**, *15*, 235-284.
7. URL http//drnelson.utmem.edu/homepage.html.
8. Maughan, J. A.; Nugent, J. H. A.; Clark, I. M.; Westlake, A. C. G.; Hallahan, D. L. *Brighton Crop Prot. Conf. Weeds*; BCPC: Farnham, UK, 1997; Vol. 2, *7A-4*, 597-604.
9. Chapple, C. *Annu. Rev. Plant Physiol. Mol Biol.* **1998**, *49*, 311-343.
10. Sariaslani, F. S. *Adv. Appl. Microbiol.* **1991**, *36*, 133-178.
11. Vanden Bossche, H.; Koymans, L. *Mycoses* **1998**, *41*, 32-38.
12. Van den Brink, H. J. M.; van Gorcom, R. F. M.; van den Hondel A. M. J. J.; Punt, P. J. *Fungal Gen. Biol.* **1998**, *23*, 1-17.
13. O'Keefe, D. P., *Drug Metab. Drug Interact.* **1995**, *113*, 755-763.
14. Blee, E.; Joyard, J. *Plant Physiol.* **1996**, *110*, 445-454.
15. Werck-Reichhart, D. *Proc. Brighton Crop Prot. Conf. Weeds*; BCPC: Farnham, UK, 1995; Vol. 3, *7B-1*, 813-821.
16. Barrett, M. *Drug Metab. Drug Interact.* **1995**, *12*, 299-316.

17. Frear, S. *Drug Metab. Drug Interact.* **1995**, *12*, 329-358.
18. Gaillardon, P.; Cabanne, F.; Scalla, R.; Durst, F. *Weed Res.* **1985**, *25*, 397-402.
19. Cabanne, F.; Huby, D.; Gaillardon, P.; Scalla R.; Durst, F. *Pestic. Biochem. Physiol.* **1987**, *28*, 371-380.
20. Gonneau, M.; Pasquette, B.; Cabanne, F.; Scalla, R. *Weed Res.* **1988**, *28*, 19-25.
21. Canivenc, M.-C.; Cagnac, B.; Cabanne, F.; Scalla, R. *Plant Physiol. Biochem.* **1989**, *27*, 1-10.
22. Fonné, R. Ph.D. thesis, University Louis Pasteur, Strasbourg, France, 1985.
23. Mougin, C.; Cabanne F.; Canivenc, M.-C.; Scalla, R. *Plant Sci.* **1990**, *66*, 195-203.
24. Mougin, C.; Polge, N.; Scalla, R.; Cabanne, F. *Pestic. Biochem. Physiol.* **1991**, *40*, 1-11.
25. Mougin, C.; Cabanne, F.; Scalla, R. *Plant Physiol. Biochem.* **1992**, *30*, 769-778.
26. Helvig, C.; Tardif, F. J.; Seyer, A.; Powles, S. B.; Mioskowski, C.; Durst, F.; Salaün, J. P. *Pestic. Biochem. Physiol.* **1996**, *54*, 161-171.
27. Shiota, N.; Inui, H.; Okhawa, H. *Pesticide Biochem. Physiol.* **1996**, *54*, 190-198.
28. Robineau, T.; Batard, Y.; Nedelkina, S.; Cabello-Hurtado, F.; LeRet, M.; Sorokine, O.; Didierjean, L.; Werck-Reichhart, D. *Plant Physiol.* **1998**, *118*, 1049-1056.
29. Siminsky, B.; Corbin, F. T.; Ward, E. R.; Fleischmann T. J.; Dewey, R. E. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 1750-1755.
30. Kemp, M. S.; Moss, S. R.; Thomas, T. H. In *Managing Resistance to Agrochemicals*; Green, M. B., Le Baron, H. M., Moberg, W. K., Eds.; ACS Symposium Series 421; ACS: Washington, DC, 1990, pp 377-393.
31. Preston, C.; Tardif, F. J.; Christopher, J. T.; Powles, S. B. *Pestic. Biochem. Physiol.* **1996**, *54*, 123-134..
32. Menedez, J.; De Prado, R. *Physiol. Plant.* **1997**, *99*, 97-104.
33. Corio-Costet, M.-F.; Benveniste, P. *Pestic. Sci.* **1988**, *22*, 343-357.
34. Corio-Costet, M.-F.; El Achouri, M.; Lanot, R.; Charlet, M.; Benveniste, P.; Hoffmann, J. *Proc. Natl. Acad. Sci. USA* **1987**, *84*, 643-647.
35. Omura, T.; Sato, R. *J. Biol. Chem.* **1964**, *239*, 2370-2385.
36. Franklin, M. R. *Pharmacol. Ther. A.* **1977**, *2*, 227-245.
37. Mougin, C.; Laugero, C.; Asther, M.; Chaplain, V. *Pestic. Sci.* **1997**, *49*, 169-177.
38. Mougin, C.; Pericaud, C.; Malosse, C.; Laugero, C.; Asther, M. *Pestic. Sci.* **1996**, *47*, 51-59.
39. Mougin, C.; Pericaud, C.; Dubroca, J.; Asther, M. *Soil Biol. Biochem.* **1997**, *29*, 9-10.
40. Barrett, M.; Avdiushko, S.; Young, A.; Polge, N.; Poneleit, C. *WSSA Abstracts* **1999**, *39*, 249.

Downloaded by OHIO STATE UNIV LIBRARIES on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch009

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch010

Chapter 10

Use of Enzymes in Bioremediation

Jerzy Dec and Jean-Marc Bollag

Laboratory of Soil Biochemistry, Center for Bioremediation and Detoxification, The Pennsylvania State University, University Park, PA 16802

Bioremediation technologies rely on the activity of microbial or plant enzymes involved in the metabolic and cometabolic transformation of a variety of organic substrates. Many xenobiotic compounds can be degraded by intracellular enzymes and thereby undergo detoxification. To date, contaminants have been exposed to enzymatic degradation primarily by stimulating microbial growth in contaminated areas. As is well known, enzymes can be active outside the microbial cells. The extracellular activity of enzymes is expected to be increasingly exploited in future bioremediation technologies. Enzymes can be obtained in large quantities from microbial populations grown under optimal conditions and without exposure to toxic chemicals. To prevent losses of enzymatic activity under severe field conditions, many investigations have been focused on developing methods to stabilize enzymes, preferably by immobilization on solid supports or by gel coating. The use of enzymes naturally stabilized in plant tissues also has been investigated. The feasibility of enzymatic treatment has been demonstrated at laboratory scale in a number of studies. For example, hydrolases from *Pseudomonas spp.* and other bacteria have been shown to hydrolyze and detoxify organophosphate pesticides. Several fungal phenoloxidases effectively oxidized xenobiotic phenols and anilines to reactive intermediates that subsequently were detoxified through polymerization or binding to humus. Further studies are necessary to identify more enzymes that may be able to transform the increasing number of chemicals polluting the environment.

In Pesticide Biotransformation in Plants and Microorganisms; Hall, J., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2000.

Introduction

To date, enzymes have found many practical applications in analytical chemistry and medicine (*1*, *2*), and now attempts are underway to employ these biological catalysts for the protection of the environment (*3*, *4*). Enzymatic processes are expected to replace many traditional industrial technologies to reduce emission of toxic by-products and to eliminate hazards associated with the use of dangerous chemicals, such as chlorine in paper bleaching or strong acids in starch processing. Enzymes also have potential for bioremediation of polluted environments.

Bioremediation is a process in which microorganisms or other biologically active agents are used to degrade environmental pollutants. The application of microorganisms to polluted areas is an established method of bioremediation (5, 6). Enzymatic treatment, however, is only at the stage of laboratory trials. The rationale for developing enzymatic cleanup methods is that enzymes are the ultimate cause of pollutant degradation during bioremediation procedures based on microbial activity. If applied extracellularly, enzymes may eliminate or at least considerably reduce the need to support specific microorganisms in the treated areas.

Enzymatic treatment is expected to have many advantages (7), such as the ease and simplicity of application, short treatment time, ability to target specific pollutants, low sludge volume, and the absence of ecological hazard associated with their use. Enzymatic treatment may have a number of advantages over microbial treatment. First, enzymes do not require an acclimatization phase as is often required by microorganisms. Enzymes can be used under a wider range of environmental conditions (e.g., pH, moisture, and temperature) than microorganisms. Unlike microorganisms, enzymes may be effective at both low and high pollutant concentrations. They can penetrate microporous sites in the soil matrix that cannot be entered by microorganisms. Enzymes are resistant to many inhibitors that may affect microbial metabolism. Furthermore, following isolation from genetically engineered microorganisms, enzymes can be released safely into the environment.

One of the drawbacks of enzymatic treatment is the high cost of isolating enzymes from microbial cells, storing them and prolonging their stability under harsh environmental or industrial conditions. Another disadvantage is the need for cofactors, without which many enzymes do not show any activity.

The most desired enzymatic action is complete mineralization of the toxic compounds. Unfortunately, as discussed in detail later, the majority of enzymes are not capable of mineralization when applied individually. Theoretically, mineralization may be possible if several different enzymes are applied simultaneously, but all enzymes that are known to be involved in the established mineralization pathways require very expensive cofactors.

With individual enzymes, one can expect only a certain rate of pollutant degradation or transformation. However, as long as the transformation products are less toxic than the parent compounds, or, in other words, as long as there is a

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch010

detoxification effect, the enzymatic treatment may be considered successful, especially when the degradation products are susceptible to further transformation by microorganisms. This and other aspects of enzymatic treatment are the subject matter of this chapter.

Enzymes Tested for Bioremediation

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch010

The idea of using extracellular enzymes for decontamination originated from research efforts to identify and characterize enzymes responsible for the transformation of xenobiotics in living organisms, mostly fungi and bacteria (*8, 9, 10, 11, 12, 13, 14*). As early as 1965, Kearney and Kaufman (*9*) reported that a soil bacterium identified as a species of *Pseudomonas* possessed an enzyme that could hydrolyze the herbicide isopropyl *N*-(3-chlorophenyl) carbamate and several other biologically active phenylcarbamates. The reaction pathway is presented in Figure 1. The authors did not view their findings in terms of bioremediation or even detoxification; they were mostly concerned with losses in herbicidal activity and wanted to identify the responsible factors. Nevertheless, their discovery was noticed and remembered by those who later focused on the decontamination aspects.

First reports that explicitly proposed the use of enzymes for cleanup appeared in scientific journals shortly after the emergence of bioremediation technologies in the 1970s. One pioneering study in this area was on the enzymatic hydrolysis of parathion, carried out by Munnecke (*15*). The enzyme was isolated from a mixed culture of soil bacteria consisting mostly of *Pseudomonas* species.

Barik and Munnecke (*16*) used a crude cell extract obtained by brief sonication of *Pseudomonas* bacteria at 150 W. The enzyme was immobilized on solid supports, such as ground Jena glass, or controlled pore glass beads. When applied to wastewater treatment, the enzyme removed 95% of parathion at initial concentrations ranging from 10 to 250 ppm. Soil treatment resulted in a complete, 100% hydrolysis of parathion that had been present at a concentration of 2500 ppm. The same enzyme showed considerable efficiency in the hydrolysis of several other organophosphate pesticides, such as triazophos, diazinon, and fenitrothion. The rate of enzymatic hydrolysis was from 11 to more than 2000 times faster than that of chemical hydrolysis (Table 1).

Munnecke (*17*) prepared a list of enzymes that showed potential for pesticide hydrolysis. The list includes mainly esterases and a few acylamidases. Johnson and Talbot (*18*) reviewed microbial enzymes, mostly hydrolases, esterases, or amidases, that are capable of transforming a variety of pesticides, such as organophosphates, carbamates, phenylureas, acylanilides, or phenoxyacetates. Few of these enzymes, however, were used to demonstrate their ability to decontaminate water or soil. Recently, Karam and Nicell (*4*) presented a list of important enzymes that had been

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch010

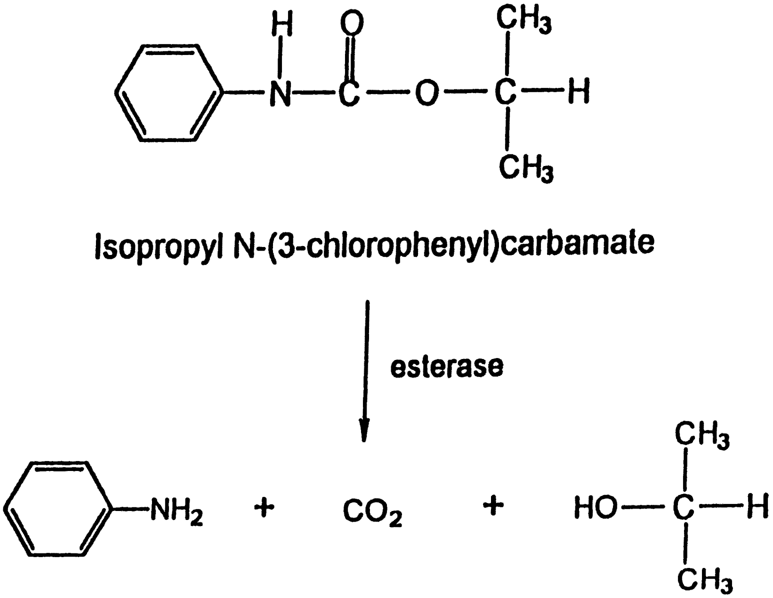


Figure 1. *Hydrolysis of a phenyl carbamate by an esterase from Pseudomonas sp. (8).*

Table 1. Enzymatic and Chemical Hydrolysis of Selected Insecticides (*15*)

Pesticide	*Enzymatic hydrolysis (nmol/min)*	*Chemical hydrolysis (nmol/min)*	*Ratio (enzymatic/chemical)*
Parathion	416	0.17	2450
Triazophos	1360	1.35	1005
Paraoxon	500	0.95	525
EPN	12	1.05	11
Diazinon	200	1.40	143
Methyl parathion	354	2.90	122
Dursban	36	0.89	40
Fenitrothion	217	1.05	205
Cyanophos	58	0.79	73

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch010

Table 2. Enzymes Studied for Their Decontamination Potential (*4*)

Enzyme	*Source*	*Target pollutant*	*Decontamination mechanism*	*Ref.*
Peroxidase (EC 1.11.1.7)	Horseradish root	Phenols Anilines	Polymerization Binding to humus	*32*
Lignin peroxidase (EC unknown)	*Phanerochaete chrysosporium*	PHA's, Phenols	Oxidation	*50*
Manganese peroxidase (EC unknown)	*Phanerochaete chrysosporium*	Phenols Anilines	Oxidation	*51*
Chloroperoxidase (EC 1.11.1.10)	*Caldariomyces fumago*	Phenols	Oxidation	*50*
Laccase (EC 1.10.3.2)	*Rhizoctonia praticola* *Trametes versicolor* *Trametes villosa*	Phenols Anilines	Polymerization Binding to humic substances	*33*
Tyrosinase EC 1.14.18.1)	Mushroom	Phenols Anilines	Polymerization	
Parathion hydrolase (EC unknown)	*Pseudomonas* sp. *Flavobacterium* sp. *Streptomyces*	Methyl parathion, ethoxy-parathion, diazinon, dursban, fensulfothion, coumaphos, potasan	Hydrolysis	*17*
Alkylsulfatase (EC unknown)	*Pseudomonas* C12B	Alkyl sulfates, alkyl ethoxy sulfates, aryl sulfonates	Complete surfactant degradation	*52*
Cyanidase (EC unknown)	*Alcaligenes denitrificans*	Cyanide	Conversion to ammonia and formate	*53*
Cyanide hydratase (EC 4.2.1.66)	*Gloeocerospora sorghi* *Stemphylium loti*	Cyanide	Hydrolysis to formamide	*54*

tested for their decontamination potential (Table 2). The list contains several oxidoreductases, the parathion hydrolase, an alkylsulfatase, a cyanidase, and a cyanide hydratase. Many enzymes have been identified as responsible for cometabolic degradation of different xenobiotics in living organisms (see the next section), but currently, they appear too costly for application in bioremediation.

Enzymes Showing the Ability to Degrade Xenobiotics

Some xenobiotics may be used by microorganisms as sources of energy and nutrients for growth. Another important mode of microbial transformation is cometabolism, in which microorganisms transform the xenobiotic molecules but are unable to proliferate on the resulting degradation products. According to literature data, oxidoreductases and hydrolases play a major role in both the metabolic and cometabolic transformation of xenobiotics (*4, 18, 19*). The decontamination potential of some enzymes is illustrated below by the transformation pathways of four common contaminants (2,4-D, atrazine, naphthalene, and 4-chlorobiphenyl).

Based on various literature data, the herbicide 2,4-D is transformed by a dioxygenase from *Alcaligenes eutrophus* to 2,4-dichlorophenol with the release of glyoxylate (*20, 21, 22*). In turn, 2,4-dichlorophenol is hydroxylated by an oxygenase and the aromatic ring of the resulting dichlorocatechol is cleaved by a dioxygenase. Eventually, a series of further enzymatic reactions leads to mineralization.

The intensely studied degradation pathway for atrazine involves the dehalogenation and hydroxylation of the herbicide by a hydrolase from *Pseudomonas* (*23*). Further steps, mediated by other hydrolases, can lead to mineralization (evolution of CO_2) via gradual hydroxylation to cyanuric acid (*24, 25*) and aromatic ring cleavage (*26*), resulting in the release of biuret and then urea (Figure 2).

Biodegradation of naphthalene may proceed through the oxidation of the compound to dihydroxynaphthalene by a dioxygenase from *Pseudomonas* sp. and then by a dehydrogenase (*27, 28*). The enzymatic hydroxylation gives rise to ring cleavage in the presence of an isomerase, and to mineralization of the resulting intermediates (salicylaldehyde and pyruvate) after they enter the general metabolic track.

Similarly, dioxygenases, dehydrogenases, and hydrolases from *Pseudomonas* sp. (*29, 30, 31*) can mediate the transformation of 4-chlorobiphenyl to aromatic or aliphatic intermediates (2-hydroxypenta-2,4-dibenzoate and 4-chlorobenzoate) that are quickly metabolized to CO_2 and water. Enzymes involved in the degradation pathways outlined above show great potential for bioremediation; unfortunately, most of them require expensive cofactors, such as NAD, NAPD or FAD.

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch010

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch010

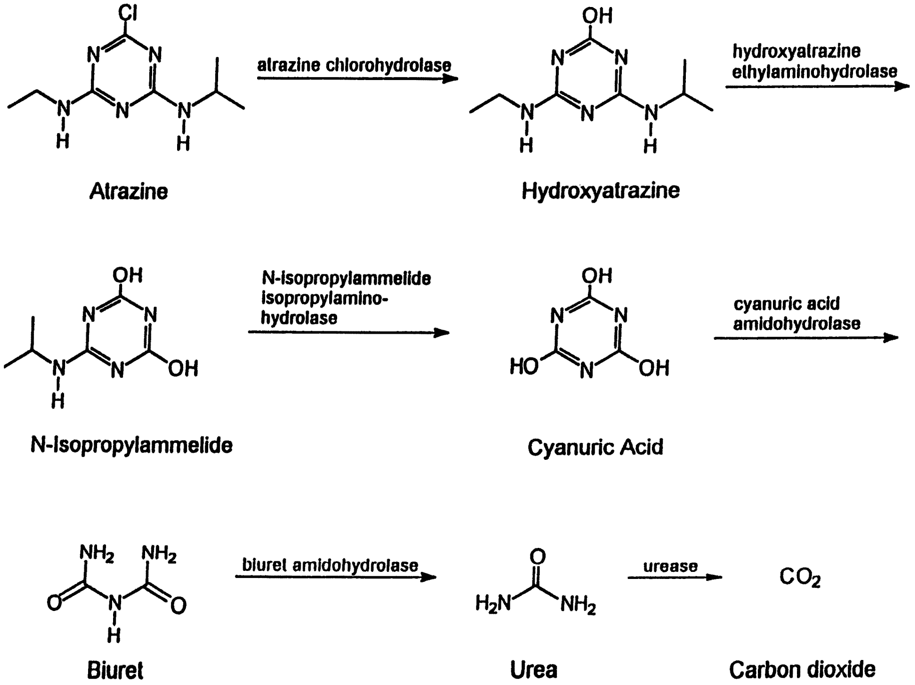

Cl
N
N
N
N
N
H
H
Atrazine
atrazine chlorohydrolase
OH
Hydroxyatrazine
hydroxyatrazine ethylaminohydrolase
N-Isopropylammelide
N-Isopropylammelide isopropylamino-hydrolase
HO
Cyanuric Acid
cyanuric acid amidohydrolase
NH2
O
Biuret
biuret amidohydrolase
H2N
Urea
urease
CO2
Carbon dioxide

Figure 2. Degradation of atrazine by various hydrolases from Pseudomonas sp. (23, 24, 26).

Decontamination of Water and Soil Using Various Oxidoreductases

Klibanov et al. (*32*) discovered that horseradish peroxidase can transform toxic phenols and anilines to nontoxic polymers that can be removed from aqueous solution by filtration or sedimentation. Similar detoxification reactions were later observed with various phenoloxidases, such as laccase or tyrosinase (*33, 34, 35*). In a study of Dec and Bollag (*36*), the removal of various chlorophenols in the presence of horseradish peroxidase ranged from 40 to 100%, depending on the number and position of the chlorine atoms. Large removals also were found with the laccase of *Trametes versicolor* (91 to 100%). Tyrosinase was less effective, especially for pentachlorophenol; nevertheless, 100% of 4-chlorophenol was removed.

Experiments using the electron spin resonance technique indicated that peroxidases and laccases oxidize substrates to free radicals that subsequently are involved in chemical coupling and oligomer formation (dimers in the first stage) (*37*). Depending on the resonance form of the free radicals, some coupling reactions were accompanied by the release of one or two chloride ions (*38*). With tyrosinase, the oligomerization resulted from the formation of *ortho*-quinones and *ortho*-hydroxylated ions that may couple to each other through nucleophilic substitution. Two routes of coupling may exist, i.e., one with and the other without release of a chloride ion (*39*).

Various phenoloxidases also may be involved in binding reactions between xenobiotics and soil organic matter. Just as with polymerization, binding presently is considered to be an efficient and safe method of detoxification (*33*). In the studies of Sarkar et al. (*40*), ^{14}C-labeled 2,4-dichlorophenol was bound to fulvic acid in the presence of various phenoloxidases (peroxidase, tyrosinase, a laccase of *Trametes versicolor*, and a laccase of *Rhizoctonia praticola*). The extent of enzymatic binding after 36 hours of incubation ranged from about 40% for tyrosinase to 70% for the laccase of *Trametes versicolor*. Experimental data indicated that, as with oligomerization, enzymatic binding may be controlled by a free radical mechanism, in which free radicals generated by the enzyme are involved in coupling with free radicals present in humic acid (*38*). Again, some coupling reactions may be accompanied by the release of chloride ions.

Stabilized Enzymes

Enzymes must be stabilized to maximize their life-times under severe environmental or industrial conditions. Immobilization is one of the most efficient approaches for stabilizing enzymes. Several mechanisms may be used to immobilize

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch010

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch010

enzymes, such as covalent attachment to solid supports, adsorption on solid surfaces, entrapment in polymeric gels, encapsulation, or intermolecular cross-linking (*41*). Solid supports used to immobilize enzymes can be divided into organic supports and inorganic supports. Organic supports may consist of synthetic matrices, such as acrylamide gels and ion-exchangers, or of natural matrices, such as lignin, humus, or cellulose. Among inorganic supports, one can use porous glass, metal oxides, and several other materials.

As already mentioned, the parathion hydrolase used by Barik and Munnecke (*16*) was immobilized on ground glass or porous glass beads. In the studies of Leonowicz et al. (*42*) and Sarkar et al. (*43*), various enzymes including phenoloxidases that mediated polymerization and binding were immobilized on clay or soil. Prior to immobilization, these supports were treated with concentrated nitric acid, and then were activated with 3-aminopropyltriethoxysilane followed by glutaraldehyde. Enzymes immobilized by this method (glucose oxidase, β-D-glucosidase, laccase, and tyrosinase) showed increased resistance to elevated temperatures as compared to free enzymes. They were also considerably less susceptible to protease activity than the free enzyme. Moreover, losses of laccase activity during a 15-day exposure to a suspension of soil were remarkably reduced after immobilization as compared to losses sustained by the free enzyme. This finding constitutes great promise for using immobilized laccases for soil treatment.

One of the most remarkable properties of immobilized enzymes is that they can be reused many times. As determined by Ruggiero et al. (*44*), laccase immobilized on different clays, such as kaolinite and montmorillonite, or on soil could be reused up to 24 times for the removal of ^{14}C-labeled 2,4-dichlorophenol from polluted water. After each 2-hour cycle the treated water was replaced with a fresh portion of the polluted water.

Use of Plant Materials Containing Peroxidase Activity

As is well known, many technological developments were inspired by nature. Immobilization of enzymes is no exception. Enzymatic activity that can be detected in all samples of fresh soil originates largely from naturally immobilized extracellular enzymes (*45*). Many plant tissues contain enzymes that by all appearances are immobilized (*46*). In an attempt to use these natural resources, Dec and Bollag (*47*) tested horseradish roots, which are known to contain large amounts of peroxidase. The experiments were carried out using horseradish cut into small pieces to decontaminate wastewater from a company that manufactured the herbicide 2,4-D. The wastewater contained up to 850 ppm of 2,4-dichlorophenol and other chlorinated phenols.

The decontamination reaction was initiated by adding hydrogen peroxide as an electron acceptor. Peroxidases that were naturally immobilized in horseradish could remove up to 100% of the initial 2,4-dichlorophenol depending on the concentration

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch010

of hydrogen peroxide. High removals were also achieved using other plant materials containing peroxidases, such as potato or white radish. As shown in the studies of Roper et al. (*48*), 55% of 2,4-dichlorophenol that had been incubated with cut horseradish precipitated from aqueous solution in the form of a polymer, and 24% was covalently bound to the plant tissue. The remainder consisted of oligomer products that either were dissolved in aqueous solution (12%) or physically sorbed to the plant tissue (8%). Horseradish application resulted in 99% removal of 27 compounds among 50 compounds tested, including eight U.S. Environmental Protection Agency priority pollutants.

An experiment for the removal of 2,4-dichlorophenol at varying pH proved that cut horseradish tissue was at least as effective as purified peroxidase obtained from Sigma (St. Louis, MO). In fact, the pH range for nearly complete removal of 2,4-dichlorophenol was greater in the presence of horseradish (pH 3 to 8) than in the presence of purified enzyme (pH 4 to 7).

Moreover, similar to enzymes immobilized on solid supports, cut horseradish could be recycled (*47*). Peroxidase naturally immobilized in horseradish tissue removed 100% of 2,4-dichlorophenol from 15 fresh portions of polluted water. Each cycle was stopped after 30 minutes of incubation. The plant material retained significant amounts of peroxidase activity (about 50%) by cycle 30, when the experiment was terminated.

Using cut horseradish, it was possible to detoxify chlorinated phenols through their binding to soil. In the study of Flanders et al. (*49*), horseradish combined with different peroxides mediated irreversible binding of 2,4-dichlorophenol to soil, immobilizing up to 92% of the pollutant. In sharp contrast, the immobilization observed in the control samples (untreated soil, or soil treated only with horseradish or peroxide) did not exceed 12%, clearly indicating that peroxidase activity present in the plant tissue was instrumental in enhancing the binding. The detoxification process was completed in 30 minutes. The immobilization of 2,4-dichlorophenol increased with increasing soil moisture. Apparently, as more water was added, contact between the pollutant and the enzyme was improved. The hydrogen peroxide was quickly decomposed in soil; therefore only 43% of 2,4-dichlorophenol was bound. With calcium peroxide, however, binding increased to 92%, probably because slow release of H_2O_2 from calcium peroxide allowed efficient utilization by the peroxidase present in horseradish. Although the effect of calcium peroxide requires further investigation, it is clear that plant materials containing peroxidases can mediate detoxification processes and, therefore, they constitute a promising remediation option.

Conclusions

Extracellular enzymes show great promise for their exploitation in future bioremediation technologies. They can be used either individually to enhance microbial degradation or in mixtures to provide for a complete mineralization of xenobiotics. The use of artificially or naturally immobilized enzymes may considerably improve the efficiency of enzymatic treatment.

Literature Cited

1. Kresse, G. B. In *Biotechnology*; Reed, G.; Nagodawithana, T. W., Eds.; VCH Verlagsgesellschaft mbH: Weinheim, Germany, 1995; Vol. 9, pp. 137-163.
2. Uhlig, H. *Industrial Enzymes and Their Applications*. John Wiley: New York, 1998.
3. Dick, W. A.; Tabatabai, M. A. In *Bioremediation of Contaminated Soils*. Adriano, D.C.; Bollag, J.-M.; Frankenberger, W. T.; Sims, R. C., Eds.; American Society of Agronomy: Madison, WI; Agronomy Monograph 37, 1999.
4. Karam, J.; Nicell, J. A. *J. Chem. Tech. Biotechnol.* **1997**, *69*, 141-153.
5. *Bioremediation of Contaminated Soils*. Adriano, D.C.; Bollag, J.-M.; Frankenberger, W. T.; Sims, R. C., Eds.; American Society of Agronomy: Madison, WI; Agronomy Monograph 37, 1999.
6. Alexander, M. *Biodegradation and Bioremediation*. Academic Press: San Diego, CA, 1999.
7. Nannipieri, P.; Bollag, J.-M. *J. Environ. Qual.* **1991**, *20*, 510-517.
8. Kearney, P. C. 1965. *J. Agric. Food Chem.* **1965**, *13*, 561-564.
9. Kearney, P. C.; Kaufman, D. D. *Science* **1965**, *147*, 740-741.
10. Brown, B. R. In *Oxidative coupling of phenols*; Taylor, W. I., Battersby; A. R., Eds.; Marcel Dekker, Inc.: New York, 1967. p.167-201.
11. Wallnöfer, P.R.; Bader, J.. *Appl. Microbiol.* **1970**, *19*, 714-717.
12. Engelhardt, G.; Wallnöffer, P. R.; Plapp, R. *Appl. Microbiol.* **1971**, *22*, 284-288.
13. Engelhardt, G.; Wallnöffer, P. R.; Plapp, R. *Appl. Microbiol.* **1973**, *26*, 709-718.
14. Mulbry, W. W.; Karns, J. S. *Appl. Environ. Microbiol.* **1989**, *55*, 289-293.
15. Munnecke, D. M. *Appl. Environ. Microbiol.* **1976**, *32*, 7-13.
16. Barik, S.; Munnecke., D. M. *Bull. Environ. Contam. Toxicol.* **1982**, *29*, 235-239.
17. Munnecke, D. M. *Process Biochem.* **1978**, *13*, 14-16,31.
18. Johnson, L. M.; Talbot, Jr., H. W. *Experientia* **1983**, *39*, 1236-1246.
19. Gianfreda, L.; Feng, Xu; Bollag, J.-M. *Biorem. J.* **1999**, *3*, 1-12.
20. Tiedje, J. M.; Alexander, M. *J. Agric. Food Chem.* **1969**, *17*, 1080-1084.
21. Fukumori, F.; Hausinger, R. P. *J. Biol. Chem.* **1993**, *268*, 2431-2437.
22. Radnoti de Lipthay, J.; Barkay, T.; Vekova, J.; Sorensen, S. *J. Appl. Microbiol. Biotechnol.* **1999**, *51*, 207-214.
23. de Souza, M. L.; Sadowsky, M. J.; Wackett, L. P. *J. Bacteriol.* **1996**, *178*, 4894-4900.

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch010

24. Boundy-Mills, K. L.; de Souza, M. L.; Mandelbaum, R. T.; Wackett, L. P.; Sadowsky, M. J. *Appl. Environ. Microbiol.* **1997**, *63*, 916-923.
25. Sadowsky, M. J.; Tong, Z.; de Souza, M.; Wackett, L. P. *J. Bacteriol.* **1998**, *180*, 152-158.
26. Cook, A. M.; Beilstein, P.; Grossenbacher, H.; Hutter, R. *Biochem. J.* **1985**, *231*, 25-30.
27. Eaton, R. W.; Chapman, P. J. *J. Bacteriol.* **1992**, *174*, 7542-7554.
28. Barriault, D.; Vedadi, M.; Powlowski, J.; Sylvestre, M. *Biochem. Biophys. Res. Commun.* **1999**, *260*, 181-187.
29. Eltis, L. D.; Hofmann, B.; Hecht, H. J.; Lunsdorf, H.; Timmis, K. N. *J. Biol. Chem.* **1993**, *268*, 2727-2732.
30. Barriault, D.; Durand, J.; Maaroufi, H; Eltis, L. D.; Sylvestre, M. *Appl. Environ. Microbiol.* **1998**, *64*, 4637-4642.
31. Hofer, B.; Eltis, L. D.; Dowling, D. N.; Timmis, K. N. *Gene* **1993**, *130*, 47-55.
32. Klibanov, A. M.; Tu, T.-M.; Scott, K. P. *Science* **1983**, *221*, 259-261.
33. Bollag, J.-M. *Environ. Sci. Technol.* **1992**, *25*, 1876-1881.
34. Wada, S.; Ichikawa, H.; Tatsumi, K. *Biotechnol. Bioengin.* **1995**, *45*, 304-309.
35. Xu, F. *Biochem.* **1996**, *35*, 7608-7614.
36. Dec, J.; Bollag, J.-M. *Arch. Environ. Contam. Toxicol.* **1990**, *19*, 543-550.
37. Suflita, J. M.; Loll, M. J.; Snipes, W. C.; Bollag, J.-M. *Soil Sci.* **1981**, *131*, 145-150.
38. Dec, J.; Bollag, J.-M. *Environ. Sci, Technol.* **1994**, *28*, 484-490.
39. Dec, J.; Bollag, J.-M. *Environ. Sci. Technol.* **1995**, *29*, 657-663.
40. Sarkar, J. M.; Malcolm, R. L.; Bollag, J.-M. *Soil Sci. Soc. Am. J.* **1988**, *52*, 688-694.
41. *Immobilization of Enzymes and Cells.* Bickerstaff, G. F., Ed.; Methods in Biotechnology 1; Humana Press: Totowa, NJ, 1997, 367 p.
42. Leonowicz, A.; Sarkar, J. M.; Bollag, J.-M. *Appl. Microbiol. Biotechnol.* **1988**, *29*, 129-135.
43. Sarkar, J. M.; Leonowicz, A.; Bollag, J.-M. *Soil Biol. Biochem.* **1989**, *2*, 223-230.
44. Ruggiero, P.; Sarkar, J. M.; Bollag, J.-M. *Soil Sci.* **1989**, *147*, 361-370.
45. Ladd, J. N.; Foster, R. C.; Nannipieri, P; Oades, J. M. In *Soil Biochemistry*; Stotzky, G.; Bollag, J.-M., Eds.; Marcel Dekker, Inc.: New York, 1996, Vol. 9, p. 23-78.
46. Mayer, A. M. *Phytochemistry* **1987**, *26*, 11-20.
47. Dec, J.; Bollag, J.-M. *Biotechnol. Bioengin.* **1994**, *44*, 1132-1139.
48. Roper, J. C.; Dec, J.; Bollag, J.-M. *J. Environ. Qual.* **1996**, *25*, 1242-1247.
49. Flanders, C.; Dec, J.; Bollag, J.-M. *Bioremed. J.* **1999**, *3*, 315-321.
50. Aitken, M. D.; Massey, J. J.; Chen, T.; Heck, P. E. *Water Res.* **1994**, *28*, 1879-1889.
51. Aitken, M. D.; Irvine, R. L. *Biotechnol. Bioengin.* **1989**, *34*, 1251-1260.
52. Thomas, O. R. T.; White, G. F. *Enz. Microb. Technol.* **1991**, *13*, 338-343.
53. Basheer, S.; Kut, Ö. M.; Prenosil, J. E.; Bourne, J. R. *Biotechnol. Bioengin,* **1992,** *39*, 629-634.
54. Basheer, S.; Kut, Ö. M.; Prenosil, J. E.; Bourne, J. R. *Biotechnol. Bioengin.* **1993**, *41*, 465-473.

Downloaded by NORTH CAROLINA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch010

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch011

Chapter 11

Transformation of Nitroaromatic Pesticides and Related Xenobiotics by Microorganisms and Plants

Robert M. Zablotowicz[1], Robert E. Hoagland[1], Hung Lee[2], T. Alber[3], Jack T. Trevors[2], J. Christopher Hall[2], and Martin A. Locke[1]

[1]Southern Weed Science Research Unit, Agricultural Research Service, U.S. Department of Agriculture, Stoneville, MS 38776
[2]Department of Environmental Biology, University of Guelph, Guelph, Ontario N1G 2W1, Canada
[3]Fachhochule Weinstephan, Fachbereich Biotechnolgie, Friesing, Germany

Nitroaromatic compounds have a range of uses including applications as pesticides and explosives. Certain nitroaromatics are common contaminants in soil and water due to their recalcitrance and wide use. Bacteria, fungi, and plants possess a wide array of enzymatic processes involved in transformation of these compounds. Bacterial oxidative mechanisms include: flavin monooxygenase mediated-nitrite release from *p*-nitrophenol, 2,4-dinitrophenol, dinitrocresol, and dioxygenase-mediated nitrite release from 2,6-dinitrophenol and nitrotoluene. Aromatic nitroreductases are ubiquitous among bacteria, certain fungi and plants. Oxidative pathways facilitate extensive degradation of nitroaromatics, however enzymes in these pathways typically have a more specific substrate range than nitroreductases. Elimination of reduced nitro groups by partial nitroreductive processes aids in subsequent metabolism of these molecules. Reductive catabolic pathways for 2,4-dinitrophenol and picric acid involve initial hydrogenation reactions. Specific plant transformations include: glutathione *S*-transferase-mediated nitrite release, nitroreductase, and ferredoxin-NADP oxidoreductase-mediated nitrite liberation. The biochemistry, physiology, and ecology of nitroaromatic metabolism are reviewed, emphasizing systems for xenobiotic detoxification.

In Pesticide Biotransformation in Plants and Microorganisms; Hall, J., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2000.

Nitroaromatic compounds are used in the synthesis of pesticides, dyes, explosives, pharmaceuticals and are of concern as environmental pollutants. Primary nitroaromatic pesticides include: dinitroaniline herbicides, e.g., pendimethalin [*N*-(1-ethylpropyl)-3,4-dimethyl-2,6-dinitrobenzenamine] and trifluralin [2,6-dinitro-*N*,*N*-dipropyl-4-(trifluoromethylbenzenamine)]; dinitrophenol herbicides, e.g., dinoseb (4-6-dinitro-*o*-*sec*-butylphenol) and 4,6-dinitrocresol (DNOC); nitrodiphenylether herbicides, e.g., acifluorfen, {5-[2-chloro-4-(trifluoromethyl)phenoxy]-2-nitrobenzoic acid}; the fungicide, pentachloronitrobenzene (PCNB); and the insecticide, parathion (*O*,*O*-diethyl-*O*-4-nitrophenol-phosphorothioate). In microorganisms, enzymes from diverse pathways metabolize nitroaromatic compounds, depending on the chemical structure and the microbial species (*1*). Likewise, numerous pathways for metabolism of nitroaromatic compounds have been described in plants. We will present some of our research on oxidative and reductive metabolism of nitroaromatic compounds by bacteria, and provide an overview on other aspects of plant and microbial transformation of nitroaromatic pesticides and related xenobiotics.

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch011

Microbial Oxidative Pathways of Nitroaromatic Metabolism

Oxidative biotransformations of numerous nitroaromatic xenobiotics have been described in diverse genera of aerobic bacteria, e.g., *Arthrobacter*, *Bacillus*, *Comamonas*, *Flavobacterium*, *Moraxella*, *Nocardia*, *Pseudomonas*, and *Sphingomonas* (Table I). With many compounds, especially more polar nitrobenzoic acids (*2*) and nitrophenols (*3*), the nitro group is enzymaticaly removed by a monooxygenase or dioxygenase, prior to ring cleavage. However, with some substrates, the nitro-group is not released until after ring cleavage, as in nitrobenzene metabolism by *Pseudomonas putida* (*4*). Certain bacteria, i.e., *Nocardia* sp. strain TW2 can degrade a nitrophenols via several pathways (*5*). When TW2 was grown (induced) on phenol, *m*-nitrophenol (MNP), or *p*-cresol, the intermediate of *p*-nitrophenol (PNP) metabolism was 4-nitrocatechol. However, when TW2 was grown on PNP, hydroquinone was the intermediate. Although numerous oxidative transformations have been implicated in the literature, the enzymes responsible for these processes have been only rarely isolated and characterized. For example, Gunderson and Jensen (*6*) isolated an *Arthrobacter simplex* strain that metabolizes DNOC, with 3-methyl-5-nitrocatechol and 2,3,5-trihydroxytoluene as intermediates, following sequential nitrite liberation.

Monooxygenases

Oxidative removal of nitro groups from *o*-nitrophenol (ONP), and PNP has been demonstrated in several bacterial strains (Table I). A *P. putida* strain was capable of metabolizing both ONP and MNP, with nitrite released from ONP and ammonium released from MNP (*3*). During nitrite release, this strain transformed ONP to catechol, which undergoes ring fission and mineralization. The enzyme, ONP-monooxygenase, has been purified and characterized (*7*). It is a soluble enzyme, that preferentially uses NADPH as the electron donor, and requires Mg^{2+} or Mn^{2+}, but doesn't require FMN or FAD. PNP transformation by a *Moraxella* sp. is also mediated by a monooxygenase that initially oxidizes PNP. This catalysis is a two-step process leading to hydroquinone with *p*-benzoquinone as the intermediate (*8*). With the *Moraxella* PNP-monooxygenase, 2 moles of NADPH are consumed during conversion of PNP to hydroquinone. This particulate enzyme required FAD, and is highly substrate specific. The *Bacillus sphaericus* PNP-monooxygenase is a two component, NADH-dependent enzyme (*9*). Component A (323 kDa) is a reductase, and component B (146 kDa) has hydroxylase functions. FAD is loosely associated with the reductase protein.

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch011

Table I. Examples of Bacterial Oxidative Nitroaromatic Transformations

Microorganism	*Compound*
Alcaligenes eutrophus	2,6-Dinitrophenol (*23*)
Arthrobacter sp.	*p*-Nitrophenol (*16*)
Arthrobacter simplex	DNOC (*6*)
Bacillus sphaericus	*p*-Nitrophenol (*9*)
Comamonas sp.	Nitrobenzene (*24*)
Flavobacterium sp.	*p*-Nitrophenol (*15*)
Moraxella sp.	*p*-Nitrophenol (*8*)
Nocardia opaca	Nitrobenzoic acids (*2*)
Pseudomonas putida	*o*-Nitrophenol and *m*-nitrophenol (*3*); *p*-nitrotoluene (*4*)
Sphingomonas sp.	*p*-Nitrophenol (*11*); 2,4-dinitrophenol, DNOC (*19*)

Flavin Monooxygenases

Certain pentachlorophenol (PCP)-metabolizing *Sphingomonas* strains can oxidatively metabolize PNP with concomitant release of nitrite (*10*). These strains degrade PCP via an initial dehalogenation reaction catalyzed by the *pcpB* gene product (PCP-flavin monooxygenase) that hydroxylates the *para* position of PCP forming tetrachlorohydroquinone (*11*, *12*, *13*). The PCP-monooxygenase can also hydroxylate the *para* position of phenolic compounds substituted with amino, nitro, nitrile and other halides; especially when the phenol also contains *ortho* substituents (*14*). The pathway for PNP degradation by *Sphingomonas* UG30 proceeds by initial hydroxylation in the *ortho* position forming *p*-nitrocatechol as an intermediate (*10*). This initial transformation is similar to that described for PNP by a *Flavobacterium* sp. (*15*) and an *Arthrobacter* sp. (*16*). Once 4-nitrocatechol is formed, it is the preferred substrate for *para* hydroxylation and nitrite release by the PCP-flavin monooxygenase. The *pcpB* gene from UG30 has been cloned and expressed in *Escherichia coli* (*17*). When the UG30 enzyme was expressed in *E. coli*, there was greater nitrite release from 4-nitrocatechol compared to PNP (*17*), confirming that

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch011

PCP-monooxygenase was not the initial enzyme in the PNP degradation pathway. This enzyme is a monomer of about 60 kDa and possesses a high degree of similarity of amino acid sequence with PCP monooxygenases from several *Sphingomonas* spp. (*17*). The PCP-monooxygenase has a highly conserved FAD binding site near the *N*-terminus that is common to other aromatic flavoprotein monooxygenases. A comparison of properties of the *Sphingomonas* PCP-4-monooxygenases and PNP monooxygenases are discussed in detail elsewhere (*18*).

Although *p*-nitrophenol is a poor substrate for the *Sphingomonas* UG30 flavin monooxygenase, this strain can metabolize certain related dinitrophenols, such as 2,4-DNP and DNOC, containing nitro groups in the *para* position (*19*). UG30, grown on glucose-glutamate broth, released a similar level of nitrite from PNP and 2,4-DNP, but lower levels of nitrite from DNOC and 4-nitrocatechol (Table II). By comparison, the highest level of nitrite release occurred with DNOC as substrate in cell suspensions of *E. coli* BL21(pBX2) induced for expression of the UG30 *pcpB* gene. An intermediate level of activity occurred with 2,4-DNP and 4-nitrocatechol as substrates and lowest activity with PNP. 2-Nitrohydroquinone and 2-methyl-6-nitrohydroquinone were identified as products of the substrates 2,4-DNP and DNOC, respectively. In UG30 cells and in the *E. coli* cloned enzyme studies, nitrite release was not observed with picric acid and dinoseb, even though both compounds possess nitro groups in the *para* position relative to the hydroxy group of the phenol ring. Apparently the butyl group in the *ortho* position of dinoseb causes either steric or electronic hindrance of this substrate for PCP-monooxygenase, while the less bulky

Table II. Comparative Metabolism of Nitroaromatic Compounds by Cultures of *Sphingomonas* sp. Strain UG30 and *Escherichia coli* BL21(pBX2) Suspensions Expressing the PCP-Flavin Monooxygenase (*pcpB*) Gene

Compound	nmole nitrite released 36 h	nmol nitrite released 6 h
	Sphingomonas UG30	*E. coli* BL21 (pBX2)
4,6-Dinitrocresol	15.6 ± 2.7*	27.3**
2,4-Dinitrophenol	66.5 ± 1.8	12.5
4-Nitrocatechol	7.5 ± 1.4	5.1
4-Nitrophenol	51.4 ± 8.9	1.2
Picric acid	< 0.5	<0.2
Dinoseb	< 0.5	<0.2
2,6-Dinitrophenol	1.4 ± 2.2	<0.2
2-Nitrophenol	1.6 ± 0.8	<0.2
3-Nitrophenol	< 0.5	<0.2
Nitrobenzene	<0.5	<0.2
p-Nitrotoluene	<0.5	<0.2
Non-inoculated	< 0.5	<0.2

Note: *Mean and standard deviation of three replicates, ** Average of two replicates
Source: Data summarized from Reference 19

ortho methyl group of DNOC resulted in the highest PCP-monoogygenase activity. Picric acid possesses a nitro group in both *ortho* positions, which may limit it as a substrate due to the electron withdrawing potenial from these two nitro groups. However, PCP with electron withdrawing chlorides in all *ortho* and *meta* positions, is a satisfactory substrate. Differences in activity of nitrophenolic substrates in whole cells of UG30 compared to the cloned enzyme, may reflect differences in inducibility of the *pcpB* gene by the different substrates, differential uptake or perhaps toxicity.

Although UG30 utilized the nitrite released from 2,4-DNP, optimum degradation of 2,4-DNP and DNOC occurred in the presence of exogenous nitrogen, especially an amino acid such as glutamate (*19*). In the absence of exogenous carbon and nitrogen 2,4-DNP was not degraded, as also observed in other bacteria (*Janthinobacterium* sp and an actinomycete) (*20*). A more rapid degradation of 2,4-DNP and accompanying nitrite release was observed when UG30 was grown in the presence of high versus low glutamate (4.0 versus 0.4g L^{-1}). However, in the presence of high glutamate, lower mineralization of the phenol ring occurred in comparison to low glutamate (8% versus 20%).

The effect of prior exposure of UG30 to 2,4-DNP, on the degradation of ^{14}C-ring labeled 2,4-DNP, was studied using methods described elsewhere (*19*). UG30 was grown on glutamate-glucose (4.0 g L^{-1} each) media, with or without 2,4-DNP. When the 2,4-DNP was depleted (72 h), cells were harvested by centrifugation and washed twice in phosphate buffer. Biometer flasks containing glutamate (0.4 g L^{-1})-glucose (4.0 g L^{-1}) broth, and a mixture of unlabeled and ^{14}C-labeled 2,4-DNP (102 μM; 1,000 Bq mL^{-1}) were inoculated with exposed or unexposed cells and incubated for 5 days. Mineralization was determined daily. Recovery of 2,4-DNP and metabolites were determined after ethyl acetate phase-partitioning and analysis by radiological thin layer chromatography (RAD-TLC) (*19*). Cultures of UG30 initially exposed to 2,4-DNP, exhibited greater mineralization, and accumulated higher levels of polar metabolites, and 2-nitrohydroquinone compared to unexposed cultures after 44 h (Table III). However, after 120 h, similar degradation and metabolite profiles were observed in exposed and unexposed cultures. These results suggest that the enzymes involved in 2,4-DNP degradation were induced by prior exposure to 2,4-DNP. Prior exposure of UG30 to PCP (inducer of *pcpB* gene expression) did not affect PNP degradation (*10*). Under both conditions a moderate amount of 2,4-DNP was mineralized (19%) and only 7% of the 2,4-DNP remained after 5 d. 2-Nitrohydroquinone, formed as a transient intermediate, and was initially higher in pre-exposed UG30 cells. Several other non-polar metabolites accumulated, with one major metabolite (Rf =0.65). We are attempting to identity this metabolite to more fully understand the 2,4-DNP metabolic pathway of UG30. Typically, nitrophenols are degraded via formation of trihydroxybenzene. Presently we do not know if release of the second nitro group occurs before or after ring cleavage, as in the case of 2,4-DNP metabolism by *Rhodococcus* (21). Possible pathways for 2,4-DNP degradation, initiated by flavin monooxygenase, PNP-monooxygenase, and a 2,4-DNP-dioxygenase are presented in Figure 1.

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch011

Table III. Recovery of Radioactivity from *Sphingomonas* sp. UG30 Cultures Treated with ^{14}C-2,4-Dinitrophenol (102 μM) after 44 and 120 h

Fraction/compound	Unexposed	2,4-DNP-exposed	Un-inoculated
	% ^{14}C recovered as		
	44 h incubation		
Mineralized (CO_2)	4.0 ± 1.0 *	9.9 ± 0.1	ND
Cellular components	1.6 ± 0.8	0.7 ± 0.1	ND
Polar metabolites	7.0 ± 0.9	19.7 ± 1.5	2.2 ± 1.4
Nonpolar compounds	80.1 ± 3.7	60.2 ± 5.7	97.5 ± 4.3
2,4-Dinitrophenol	60.8 ± 9.9	49.1 ± 6.0	97.5 ± 4.3
Rf = 0.65	14.3 ± 9.5	9.5 ± 3.7	ND
2-Nitrohydroquinone	3.1 ± 1.8	7.1 ± 1.7	ND
Other nonpolar compounds	2.0 ± 3.6	3.6 ± 1.2	ND
	120 h incubation		
Mineralized (CO_2)	18.9 ± 2.9	19.5 ± 1.6	ND
Cellular componens	6.2 ± 1.8	4.9 ± 2.0	ND
Polar metabolites	17.4 ± 0.8	20.2 ± 5.8	0.3 ± 0.1
Nonpolar compouns	47.6 ± 9.7	42.0 ± 7.1	98.8 ± 0.9
2,4-Dinitrophenol	6.5 ± 4.0	7.1 ± 5.9	98.8 ± 0.9
Rf = 0.65	27.0 ± 7.8	25.2 ± 2.5	ND
2-Nitrohydroquinone	ND	ND	ND
Other nonpolar compounds	14.1 ± 1.2	10.0 ± 3.1	ND

Note * Mean and standard deviation of three replicates, ND = none detected.

Other Oxidative Pathways

Toluenes are metabolized by certain bacteria (*4*, *22*). Studies evaluating the substrate range of nitrotoluenes in toluene-degrading *P. putida* and *E. coli* expressing *xylMA*, indicate that these genes enable oxidation of *m*-nitrotoluene and *p*-nitrotoluene to their corresponding alcohols and benzaldehydes (*22*). Benzyl alcohol and benzaldehyde dehydrogenases from *P. putida* utilize the *meta* and *para* substituted substrates, but not *o*-nitrobenzyl alcohol or 3,4-nitrobenzyl alcohol. These studies suggest a toluene dioxygenase in the metabolism of nitrotoluenes by *Pseudomonas* sp. JS150 and *P. putida* F1 (*22*). When grown on glucose, no oxidation of 2-, 3- or 4-nitrotoluene was observed, but all three compounds were metabolized when the *Pseudomonas* strains were grown on toluene. When grown on toluene, oxidation of 2-nitrotoluene to 2-nitrobenzyl alcohol occurred in both strains. With 3-nitrobenzene as substrate, JS150 formed 3-nitrobenzyl alcohol and 3-nitrobenzoate, while *P. putida* F1 formed only 3-nitrobenzyl alcohol. Both JS150 and *P. putida* F1 oxidzied 4-nitrotoluene

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch011

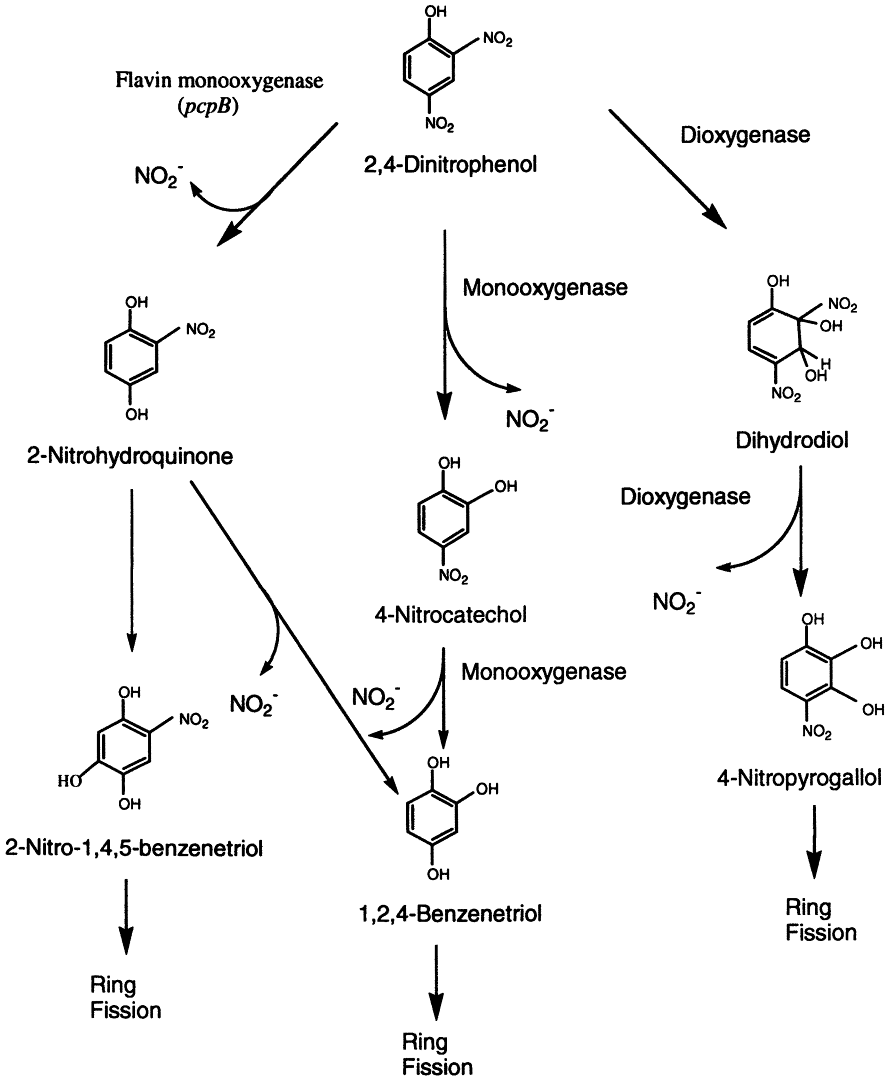

Flavin monooxygenase
(pcpB)
NO2-
2,4-Dinitrophenol
Dioxygenase
Monooxygenase
NO2-
2-Nitrohydroquinone
4-Nitrocatechol
Dihydrodiol
Dioxygenase
NO2-
NO2-
NO2-
Monooxygenase
2-Nitro-1,4,5-benzenetriol
1,2,4-Benzenetriol
4-Nitropyrogallol
Ring Fission
Ring Fission
Ring Fission

Figure 1. Metabolism of 2,4-dinitrophenol initiated by Sphingomonas sp. UG30 PCP-flavin monooxygenase (pcpB), o-nitrophenol-monooxygenase, or by dioxygenase. (Adapted from Reference 18, copyright Stockton Press.)

to 2-methyl-5-nitrophenol (major product) and 3-methyl-6-nitrocatechol (minor product). The role of the toluene dioxygenase was demonstrated in *E. coli* expressing the *todC1C2BA* genes, which oxidized the methyl substituent. These reactions showed that a dioxygenase can express monooxygenase activity with 2- and 3- nitrotoluene. Studies on the oxidation of 4-nitrotoluene with $^{18}O_2$ indicated that both oxygen atoms

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch011

were incorporated into the ring during formation of 3-methyl-6-nitrocatechol. A dioxygenase has also been implicated in catabolism of 2,6-DNP by *Alcaligenes eutrophus* (*23*). The *A. eutrophus* dioxygenase liberated nitrite from 2,6-DNP

Most bacteria metabolize nitrobenzene using a partial reduction process. However, an oxidative pathway of *Comamonas* strain JS765 (*24*), enables the use of nitrobenzene as a sole carbon and nitrogen source. Initial metabolism is via formation of a nitrodihydrodiol that decomposes to catechol and nitrite. The genes encoding for this catechol 2,3-dioxygenase have been cloned and sequenced from JS765 (*25*). The nitrobenzene dioxygenase is similar to 3-methylcatechol dioxygenase from the toluene (monooxygenase) degradation pathway of *P. picketti.*

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch011

Microbial Reductive Nitroaromatic Metabolism

Genera of aerobic (*Bacillus*, *Enterobacter*, *Escherichia*, *Mycobacterium*, *Nocardia*, and *Pseudomonas*) and anaerobic (*Bacteroides* and *Clostridium*) bacteria possess aromatic nitroreductases that catalyze the reduction of various nitroaromatic compounds to their corresponding amino derivatives (Table IV). Both polar compounds, e.g., nitrobenzoic acids (*26*) and highly nonpolar compounds, e.g., trifluralin (*27*), are subject to transformation by nitroreduction. These nitroreductases are flavoproteins that use either NADH or NADPH as reducing equivalents, require FMN or FAD as cofactor, and have varying degrees of sensitivity to oxygen. Certain organisms have multiple aromatic nitroreductase isozymes. *Bacteroides fragilis* possesses four distinct enzymes ranging in molecular weight from 52 to 680 kDa, each expressing different substrate specificity (*28*). *E. coli* possesses two nitroreductases: one oxygen sensitive and the other oxygen insensitive (*29*). A 2,4,6-TNT aromatic nitroreductase has been purified from *Enterobacter cloacae* (*30*), and the gene that encodes this enzyme has been cloned and sequenced (*31*). The *E. cloacae* nitroreductase is an oxygen-insensitive monomer of 27 kDa, requiring FMN as co-factor, and NADH or NADPH as reducing equivalents. The *E. coli nfnB* gene has also been cloned and characterized (*32*) and is very similar to the nitroreductase genes from *E. cloacae* and *Salmonella typhimurium*.

Partial reductive nitroreductases are involved in certain nitroaromatic transformations, in which the nitro moiety is initially reduced to a hydroxylamino group. This metabolite is subject to further action by a hydroxylaminolyase, e.g., *p*-nitrobenzoate degradation by *Pseudomonas picketii* (*33*). The metabolic fate of a nitroaromatic compound is dependent upon environmental conditions, the nature of the microbial community, and chemical structure characteristics that determine its susceptibility to enzymatic processes.

In addition to biological transformations, chemical reductions of nitro groups have also been reported. Humic compounds can provide protons for the reduction of trifluralin (*34*). Fe^{2+} can mediate the reduction of diphenylether herbicides in soil under anaerobic conditions (*35*). Microbial activity is responsible for the generation of the reductive conditions and formation of compounds associated with chemical nitroreduction. Therefore, it is difficult to separate strict biochemical transformations

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch011

Table IV. Examples of Reductive and Partial Reductive Transformation of Nitroaromatic Compounds by Bacteria and Fungi

Compound	*Microorganism*
	Complete aromatic nitroreduction
Acifluorfen	*Bacillus* sp. (37) *Enterobacter cloacae, P. fluorescens* and *P. putida* (39)
1-Chloro-4-nitrobenzene	*Rhodosporidium* sp. (45)
Dinitrocresol	*Azotobacter, Rhizobium,* and *Bradyrhizobium spp.* (41)
Dinitrophenol	*Rhodobacter capsulatus* (46)
p-Nitrobenzoic acid	*Nocardia* sp. and *P. fluorescens* (26)
Pendimethalin	*Fusarium oxysporum* and *Paecilomyces varioti* (44)
Trifluralin	*Streptomyces* sp. (27)
2,4,6-Trinitrotoluene	*Enterobacter cloacae* (30)
	Partial aromatic nitroreduction
2-Chloro-5-nitrophenol	*Ralstonia eutropha* (60)
p-Nitrobenzoic acid	*Comamonas acidovarans, (52)* *Pseudomonas picketii* (33)
Nitrobenzene	*Pseudomonas pseudoalcaligenes* (54)
m-Nitrophenol	*Ralstonia eutropha* (59)
4-Nitrotoluene	*Mycobacterium* sp. (58) *Pseudomonas* sp. (57)
2,4,6-Trinitrotoluene	*P. pseudoalcaligenes* (56)

from chemical nitroreduction processes. Pendimethalin is more rapidly degraded under anaerobic flooded soil conditions compared to aerated non-flooded conditions (*36*), and reduction occurs under sterile and non-sterile conditions. Under aerobic conditions, both the mono-amino derivative and the *N*-dealkylated metabolite were observed, while under flooded conditions *N*-(1-ethylpropyl)-5,6-dimethyl-7-nitrobenzimidazole was the major metabolite. The authors postulated that *N*-(1-ethylpropyl)-5,6-dimethyl-7-nitrobenzimidazole was formed from the amino-derivative (*36*).

Aromatic Nitroreduction of Herbicides

Nitroreduction of diphenylether herbicides by pure bacterial cultures was reported for acifluorfen (*37*, *38*, *39*) and 2,4-dichlorophenyl-4'-nitrophenyl ether (*40*). Nitroreduction was the major transformation reaction of acifluorfen, yielding aminoacifluorfen in enrichment cultures and pure cultures under anaerobic (*37*) and aerobic (*38*) conditions. Cell-free extracts (CFE) of *E. cloacae* and *P. fluorescens* strains possess an aromatic nitroreductase capable of reducing acifluorfen to aminoacifluorfen (*39*). Several genera of nitrogen fixing bacteria, e.g., *Azotobacter*, *Rhizobium*, and *Bradyrhizobium* spp. transformed DNOC under aerobic conditions, to form 3-amino-5-nitro-*o*-cresol as a major metabolite (*41*). Further metabolism of DNOC was observed by some strains; however, metabolites were not identified in that study.

Aminoacifluorfen-reducing bacterial strains (*P. fluorescens*, *P. putida*, and *E. cloacae*) and PNP-oxidizing strains (*Moraxella* and *Sphingomonas* sp. UG30) were evaluated for transformation of trifluralin and dinoseb under aerobic and microaerophilic conditions. Trifluralin metabolism was assessed using two methodologies. Cell suspensions (optical density = 8.0 at A=660 nm) were treated with ^{14}C-ring labeled trifluralin (20 μM), and incubated at 30°C under static conditions (microaerophilic) or shaken at 125 rpm (aerobic). After 24 and 48 h, cells were extracted with 3 volumes of acetone by sonication, then centrifuged (12,000 x g, 10 min), and the supernatant analyzed via TLC. Silica gel plates were developed with benzene:carbon tetrachloride (40:60) and hexane:methanol (97:3) solvents (*42*). The distribution of radioactivity was determined by linear imaging scanning as described elsewhere (*39*). In the second method, cell suspensions were treated with ethanolic solutions of unlabeled trifluralin (20 μM) and incubated at 28°C under static conditions for 24 h. The reaction was terminated by acidification and phase-partitioned with hexane. Hexane fractions were filtered (2 μm) and analyzed by GC-MS (Hewlett Packard 5971 GC/MS equipped with a DB-5 column). In a similar fashion, cell suspensions were treated with dinoseb (200 μM) and incubated at 30°C under static or well-agitated conditions for 48 h. Dinoseb metabolism was assessed using HPLC and GC-MS. For HPLC analysis, cells were extracted with 2 volumes of methanol, centrifuged and the supernatant filtered (2 μm). Extracts were analyzed using a Shimadzu HPLC equipped with a C-18 reverse-phase column (Phenomenex Spherex 5 μm), and a binary gradient of a) 10% terahydrofuran in methanol and b) 1% acetic acid in water (*43*). For GC/MS analysis, culture supernatants were acidified, concentrated by C-18 solid phase extraction, eluted in ethyl acetate and analyzed as described elsewhere (*19*).

All strains reduced ^{14}C-trifluralin to the mono amino derivative (Rf = 0.59, benzene:carbon tetrachloride solvent; Rf = 0.46 hexane:methanol solvent), but only under microaerophilic conditions (Table V). All three *Pseudomonas* strains accumulated polar metabolites that were immobile in both solvent systems (i.e., remained at the origin). GC-MS methodology verified formation of the monoamino-derivative as the only detectable metabolite [base peak= 305 atomic mass units (amu) (Figure 2b)] compared to trifluralin, [base peak=335 (Figure 2a)]. The greatest trifluralin reducing activity was found in *P. fluorescens* strain UA5-40 and *E. cloacae* strain ATCC43560, and *Moraxella* sp., and *Sphingomonas* UG30. had the lowest.

All strains also metabolized dinoseb under static conditions, forming an orange colored metabolite with a retention time of 7.8 min, which was not formed by any strain under aerated conditions (Table VI). GC-MS verified formation of one major metabolite, a monoamino-derivative of dinoseb (base peak of methylated metabolite =222 amu), compared to a base peak of 252 amu for the methylated parent (data not shown). The greatest nitroreductive activity on dinoseb was also observed in *E.*

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch011

Table V. Microaerophilic Metabolism of Trifluralin by Bacterial Cell Suspensions after 48 h with ^{14}C-Trifluralin, and 24 h with Unlabeled Trifluralin

	^{14}C-RAD-TLC Study			*GC-MS Study*	
	% Recovered as				
Bacterial Strain	*Trifluralin*	*Amino-trifluralin*	*Other*	*Trifluralin*	*Amino-trifluralin*
P. fluorescens, UA5-40	16.4 ± 5.9[1]	62.1 ± 3.8	22.5 ± 7.2	35.6 ± 2.5	23.8 ± 2.5
P. fluorescens, RA-2	51.3 ± 2.6	34.6 ± 3.8	12.6 ± 2.5	46.3 ± 2.5	14.4 ± 1.3
P. putida, M-17	55.7 ± 4.2	29.8 ± 3.2	14.5 ± 4.8	45.6 ±3.2	18.1 ± 1.9
E. cloacae, 43560	44.9 ± 8.0	50.0 ± 5.0	5.0 ± 5.4	48.9 ±7.5	19.4 ± 1.3
Moraxella sp.	70.2 ± 2.5	26.7 ± 2.5	2.1 ± 2.0	61.9 ± 0.7	10.6 ± 0.7
Sphingomonas sp. UG30	85.6 ± 2.8	12.0 ± 2.5	2.4 ± 2.3	75.6 ± 1.3	8.1 ± 1.3
Un-inoculated	100	ND	ND	96.0 ± 2.5	ND

Note Radiological assays, determined after acetone extraction; GC-MS analyses, determined on ethyl acetate phase-partitioned fraction. [1] Mean and standard deviation of three replicates; ND = none detected.

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch011

cloacae strain ATTCC 43560 and *P. fluorescens* strain UA5-40, and the lowest activity was in the *Moraxella* and *Sphingomonas* strains.

These studies indicate that the potential for aromatic nitroreduction of the dinitroaniline and dinitrophenol herbicides is widely distributed among several genera of gram-negative bacteria. These studies also demonstrate that multiple pathways for nitroaromatic transformation can occur in certain microorganisms, e.g., oxidative and reductive. Transformation of the trifluralin analogue, 2,6-dinitro-4-(trifluoromethyl) benzeneamine, by nitroreduction has also been shown in a *Streptomyces* sp. strain (*27*). The monoamino-derivative was the dominant metabolite in *Streptomyces*, however, this metabolite was subject to further transformation by acetylation. A summary of potential microbial nitroreductive transformations of trifluralin is outlined in Figure 3.

CFEs from 48 h tryptic soy broth cultures of *P. fluorescens* strains RA-2 and UA5-40 were prepared by sonication, centrifugation and filtration. CFEs from these strains possessed activity on several substrates *p*-nitrophenol, *p*-nitrobenzoic acid and dinoseb in addition to acifluorfen. No activity was found in CFEs with trifluralin (data not shown). Whole cell activities with the polar substrates are several-fold higher than trifluralin-nitroreductase activity. Our inability to detect trifluralin-nitroreductase activity in CFEs is most likely due to the comparatively lower solubility of trifluralin and its potential for non-specific binding to other proteins in the CFEs.

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch011

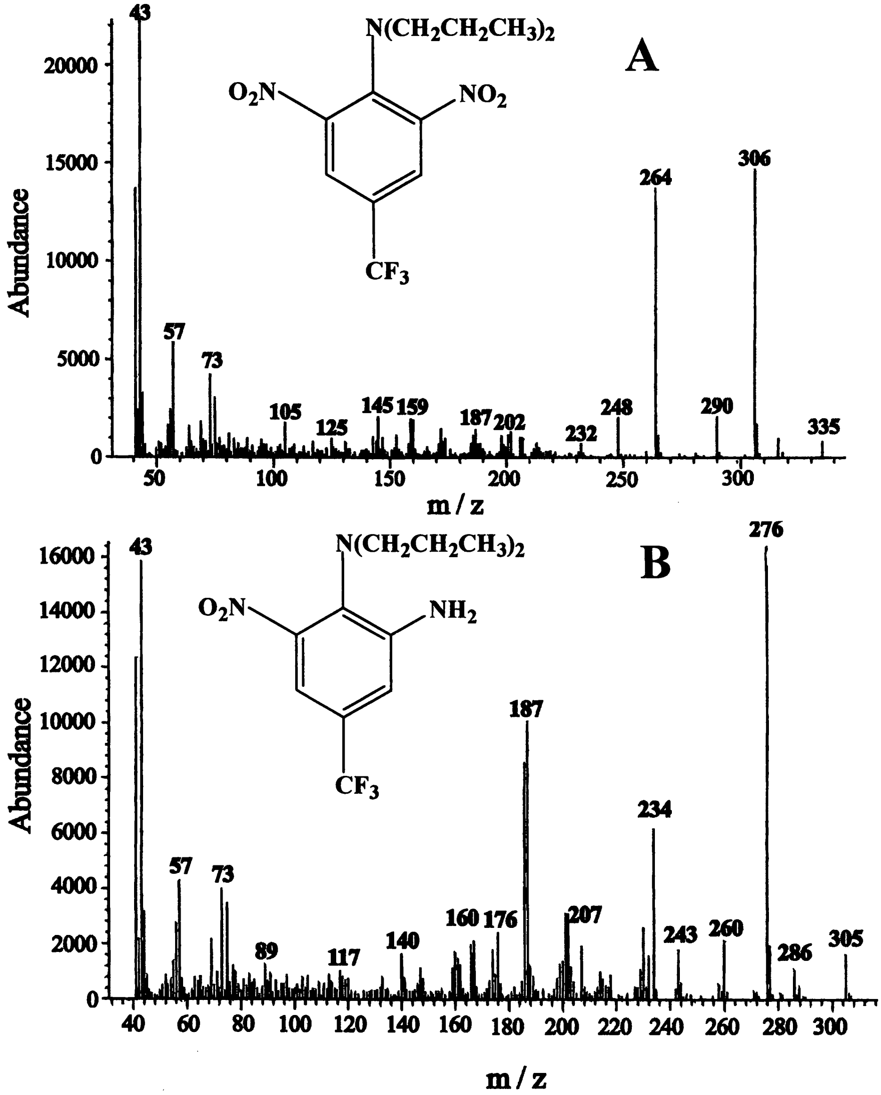


Figure 2. GC-MS spectrum of trifluralin (A) and the amino-derivative metabolite (B).

Table VI. Microaerophilic Metabolism of Dinoseb by Resting Cell Suspensions, HPLC Analysis after 48 h Incubation

Strain	Dinoseb (% Initial)[1]	Amino-derivative, (area 268 nm)[2]
Enterobacter cloacae, ATTC 43560	18 ± 9 [2]	294 x 10^3 ± 95 x 10^3
Pseudomonas fluorescens, UA5-40	38 ± 7	248 x 10^3 ± 47 x 10^3
Pseudomonas fluorescens, RA-2	41 ± 5	225 x 10^3 ± 51 x 10^3
Pseudomonas putida, M-17	52 ± 7	198 x 10^3 ± 43 x 10^3
Moraxella sp.	58 ± 9	173 x 10^3 ± 41 x 10^3
Sphingomonas sp., UG30	77 ± 8	111 x 10^3 ± 24 x 10^3
Un-inoculated	98 ± 5	27 ± 15 x 10^3

[1] Mean and standard deviation of three replicates
[2] Peak area; retention time = 7.5 min.

CFEs were concentrated via membrane filtration (20 kDa cutoff) and subjected to non-denaturing polyacrylamide gel electrophoresis. Nitroreductases were visualized on the gels after 2 h incubation in buffer [acifluorfen (80 μM), NADH (300 μM) and FMN (10 μM)] followed by diazotization (44). Both strains possessed a single nitroreductase that was highly mobile (Rf ~0.8).

Aromatic nitroreduction has been reported in several fungi. *F. oxysporum* and *Paecilomyces varioti* reduced pendimethalin to *N*-(1-ethylpropyl)-3,4-dimethyl-2-nitrobenzene-1,6-diamine in pure culture, while transformation by *Rhizoctonia solani* was via *N*-dealkylation (*44*). The yeast, *Rhodosporidium* sp., reduced 1-chloronitrobenzene to 1-chloroaniline (*45*). Nitroreduction of 2,4-DNP has been observed in the phototropic purple bacteria *Rhodobacter capsulatus* (46). Formation of 2-amino-4-nitrophenol in *R. capsulatus* cultures was observed only under low oxygen conditions. This process also required light, and was found to be inducible. This photoreduction of 2,4-DNP may be similar to the ferredoxin-mediated photoreduction of 2,4-DNP to 2-amino-4-nitrophenol which occurs in chloroplasts (*47*).

Metabolism of an aromatic compound via oxidative or partial reductive processes may be more desirable than complete nitroreduction, since the resulting metabolite is then susceptible to ring cleavage. Thus, the formation of an amino-substituted metabolite may be considered wasteful in terms of metabolic energy. In some cases, the amino derivative can be more toxic than the nitroaromatic parent, i.e., aminopyrene versus nitropyrene (*48*), and the mutagen aminodinitrotoluene formed from 2,4,6-TNT by *P. aeruginosa* (*49*). However, in soil, amino aromatic compounds are prone to oxidative coupling reactions and can be subsequently detoxified via humification. For example, when the nitro group of acifluorfen is reduced to aminoacifluorfen, the metabolite is subject to more extensive sorption and incorporation into soil humic materials (*50*, *39*). A reductive process for DNOC, involving a sequential reduction of the nitro groups to amino groups, has been reported in a *Pseudomonas* sp. (*51*). However, the amino groups are subsequently

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch011

hydroxylated, forming 2,3,5-trihydroxytoluene as the intermediate, before ring cleavage.

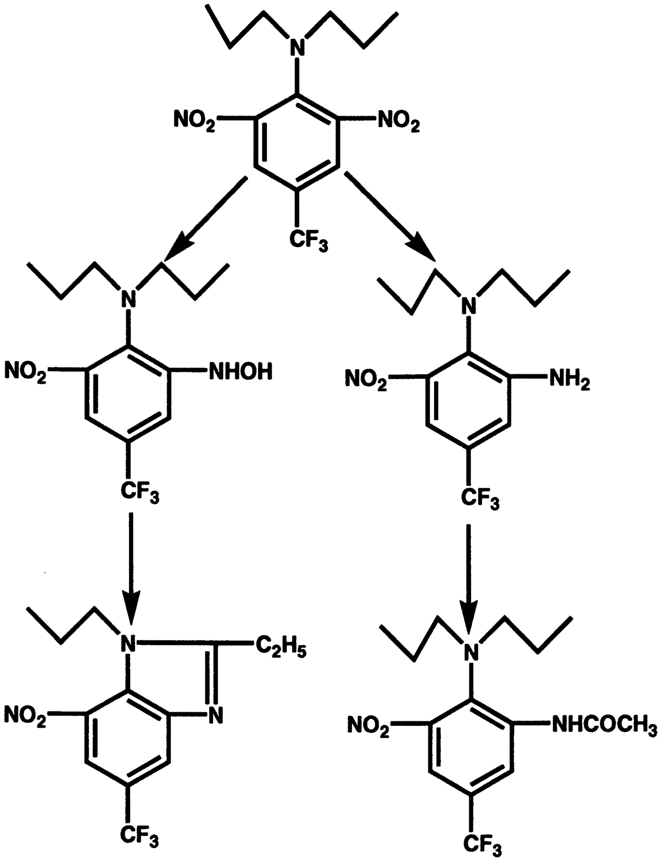


Figure 3. Microbial metabolic transformations of trifluralin resulting from initial aromatic nitroreduction.

Partial Aromatic Nitroreduction

During the last ten years numerous bacteria have been described that are capable of degrading nitroaromatic compounds via partial nitroreduction mechanisms (Table IV). Metabolism of *p*-nitrobenzoate by *Comamonas acidovarans* occurs via reduction to *p*-hydroxybenzoate, which when acted on by hydroxylaminolyase, releases ammonium and protocatechuate (*52*, *53*). The nitrobenzoate nitroreductase from *C. acidovarans* is a monomer (~ 30 kDa) and the hydroxylaminolyase has a molecular weight of 45 kDa (*53*). A similar pathway has been observed in *P. picketii*, and genes for *p*-

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch011

nitrobenzoate reductase (30 kDa) and hydroxylaminolyase (22 kDa) have been cloned from this bacterium (*33*).

Degradation of nitrobenzene by *Pseudomonas pseudoalcaligenes* strain JS52 occurred with 2-aminophenol as an intermediate (*54*). In this strain, nitrobenzene is reduced to hydroxylaminobenzene by an inducible enzyme, consuming 2 mol of NADPH. Hydrolylaminobenzene is converted to aminophenol via a Bamberger rearrangement catalyzed by hydroxylaminolyase (Figure 4). This nitrobenzene nitroreductase from *P. pseudoalcaligenes* has been purified and characterized as a monomeric flavoprotein with a molecular weight of 30 kDa, containing 2 mol FMN per mol protein (*55*). Nitrosobenzene was not detected as an intermediate of nitrobenzene metabolism, however, nitrosobenzene could serve as substrate, with hydroxylaminobenzene as the final product. 2,4,6-TNT is also a substrate for *P. pseudoalcaligenes* strain JS52 when grown on nitrobenzene (*56*). The nitrobenzene nitroreductase was capable of reducing 2,4,6-TNT to 4-hydroxylamino-2,6-dinitrotoluene, 2-hydroxylamino-4,6-nitrotouene and 2,4,-dihydroxyamino-6-nitrotoluene. This novel transformation of 2,4,6-TNT may offer promise in developing superior inoculant strains for remediating 2,4,6-TNT in contaminated soils and water.

Pseudomonas sp., strain 4-NT, utilizes 4-nitrotoluene as a sole carbon and nitrogen source (*57*). This bacterium initially oxidizes 4-nitrotoluene to nitrobenzoate. The nitro group is subsequently reduced to 4-hydroxylaminobenzoate, facilitating ammonia release and formation of protocatechuate, as exhibited in *Comamonas* metabolism of *p*-nitrobenzoate (discussed above). *Mycobacterium* sp. strain HL 4-NT-1 uses an alternative pathway in which 4-nitrotoluene is initially reduced to 4-hydroxyaminotoluene, and then undergoes rearrangement of the hydroxyl group to form 6-amino-*m*-cresol (*58*). Under anaerobic conditions 6-amino-*m*-cresol is the final product, but under aerobic conditions, the ring is hydroxylated at the *ortho* position, facilitating ring cleavage prior to ammonia release.

Ralstonia eutropha strain JMP134, utilizes 3-nitrophenol as sole carbon and nitrogen source (*59*). A nitroreductase initially reduces 3-nitrophenol to 3-hydroxylaminophenol which is then converted to aminohyroquinone prior to ring cleavage. This strain can also metabolize 2-chloro-5-nitrophenol via nitroreduction, and the chlorine group is removed by reductive dehalogenation (*60*). This *R. eutropha* nitroreductase displays partial reductive activity on a wide range of compounds including: nitrobenzene, picric acid, 3- and 4-nitrobenzoate, 2,4,6-TNT, and other mono and dinitrotoluenes (*60*). Low activity is observed with 4-nitrophenol, 2,4-dinitrophenol and 2,6-dinitrophenol.

Bacterial Hydrogenation Reactions

Rhodococcus erythropolis strains that utilize 2,4-DNP as a sole nitrogen or carbon source have been characterized (*21*). These strains metabolized 2,4-dinitrophenol, with a concomitant release of nitrite and the formation of 4,6-dinitrohexanoate as a minor dead-end product, with no further metabolism. Catabolism of 2,4-DNP by *R.*

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch011

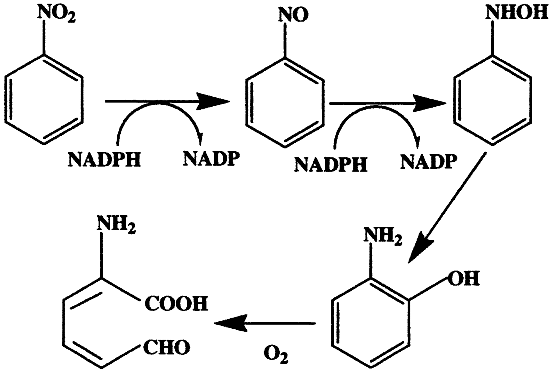


Figure 4. Pathway for transformation of nitrobenzene initiated by partial nitroreduction in Pseudomonas pseudoalcaligenes strain JS52. (Adapted from reference 54.)

erythropolis was not repressed by inorganic nitrogen. The nitrogen from 2,4-dinitrophenol was preferentially used versus inorganic nitrogen. Mutants of *R. erythropolis* strain HL 24-2 catabolized picric acid via initial hydrogenation and formation of an hydride-Meisenheimer complex (*61*). During picric acid metabolism, 2,4,6-trinitrocyohexanone accumulated as a minor terminal metabolite.

Picric acid was used as sole carbon and nitrogen source by *Nocardiodes* strains related to *Nocardiodes simplex* (*62*). In these *Nocardiodes* strains, 2,4-DNP accumulated as an intermediate and a stoichiometric release of three mol nitrite was observed for each mol picric acid. No accumulation of 2,4,6-trinitrocyohexanone was found. In more recent studies, hydride-Meisenheimer complexes of picric acid and 2,4-DNP were observed as intermediates during the mineralization of picric acid by *Nocardiodes* sp. strain CB 22-2 (*63*). These results indicate that hydrogenation reactions occur among several genera of bacteria.

Substituted dinitrophenols, e.g., 2-chloro-4,6-DNP, 2-amino-4,6-DNP and DNOC, are subject to reductive hydrogenation reactions by *R. erythropolis* strains (64). Under aerobic conditions, 2-chloro-4,6-DNP was utilized as a sole carbon and nitrogen source. Under anaerobic conditions, reductive dechlorination produced 2,4-DNP as an intermediate. During DNOC metabolism by *R. erythropolis*, the methyl group at the *ortho* position blocked ring cleavage, and 4,6-dinitro-2-methylhexanoate accumulated (*64*). 2,4,6-TNT is also subject to hydrogenation reactions by *R. erythropolis* and *Mycobacterium* sp. HL 4NT-1. Although the hydride-TNT protonated complex is formed, neither nitrite elimination nor rearomatization occurs (*65*).

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch011

Other Microbial Reductive Transformations of Nitroaromatics

The anaerobic bacterium, *Clostridium bifermentans* rapidly degrades dinoseb (66 Hammill and Crawford, 1996) under anoxic conditions. Degradation occurred via co-metabolism and a fermentable carbon source was required. Although dinoseb was degraded completely, no aromatic metabolites were detected, and mineralization to CO_2 was low (7%). Sequential reduction of 2,4,6-TNT was observed in *C. bifermentans* (*67*) and the sulfidogenic bacterium *Desulfovibrio* sp. (*68*). Reduction of one of the initial *o*-nitro and *p*-nitro groups proceeds relatively rapidly, however reduction of the final *o*-nitro group is rate limiting. In both *Desulfovibrio* and *C. bifermentans* 2,4-Diamino-4-nitrotoluene reduction was catalyzed by a ferredoxin-reducing enzyme. The end-products also differ among these two genera. The Desulfovibrio sp. reductively eliminated the amino groups forming toluene. In *C. bifermentans*, hydrolytic deamination produced 2,4,6 trihydroxytoluene, and then *p*-cresol as the metabolite prior to ring cleavage.

A collection of fungi (91 strains representing 32 genera) was assessed for the potential to transform or mineralize 2,4,6-TNT (*69*). All fungal strains reduced 2,4,6-TNT to amino derivatives, with greater accumulation occurring in micromycetes compared to basidomycetes. Mineralization (up to about 40%) was only observed in basidomycetes. The white rot fungus, *Phanerochaete chrysosporium*, transforms 2,4,6-TNT under aerobic conditions (*70*). Initial products of TNT metabolism are 2- and 4-amino-DNT (*71*). Initial studies indicated that *P. chrysosporium* does not possess nitroreductases (*72*). But, a plasma-membrane-dependent redox system (proton pump) was postulated as the mechanism for nitroreduction by *P. chrysosporium* (*73*). Following nitroreduction, 2-amino and 4-amino-DNT were mineralized to CO_2 by manganese peroxidase and lignin peroxidase (*73*). However, an intermediate of nitroreduction, 4-hydrolxylamino-2,6-DNT, is inhibitory to lignin and manganese peroxidases of *P. chrysosporium* (*74*). An oxygen sensitive, NAD(P)H, membrane-associated nitroreductase has been observed in cell-free extracts of *P. chrysosporium* by other researchers (*75*). Activity was detected on several substrates: 1,3-dinitrobenzene, 2,4-dinitrotoluene, 1-chloro-2,4-dinitrobenzene, 2,4,6-TNT. In addition, 1-nitroso-3-nitrobenzene and 1-hydroxylamino-3-nitrobenzene were utilized by this nitroreductase, indicating that these compounds may be intermediates in 1,3-dinitrobenzene metabolism.

Plant Nitroaromatic Transformations

Relative to microorganisms, there are relatively few reports on enzymatic transformations of nitroaromatic compounds in plants. The dinitroaniline herbicides are the most widely used group of nitroaromatic herbicides. Most of the available literature on their metabolic fate in plants is from the late 1960s to the early 1970s. In carrot (*Daucus carota* L.), most trifluralin residues occurred in the outer cuticular

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch011

layer. The bulk of the residues was trifluralin and low amounts of the major metabolite, *N*-depropylated trifluralin (*76*). When peanut (*Arachis hypogaea* L.) leaf extracts were exposed to ^{14}C-labelled trifluralin, the major metabolite was again, *N*-depropylated trifluralin (*34*). However, the dominant metabolite in sweet potato (*Ipomoea batatas* L.) leaf extracts was the monoamino-derivative of trifluralin (*34*).

The fungicide pentachloronitrobenzene (PCNB) is subject to three distinct initial metabolic reactions in plants (Figure 5): aryl nitroreduction, nucleophilic displacement of the nitro group via glutathione conjugation, and nucleophilic displacement of chlorines via gluathione conjugation (*77, 78*). In mammalian systems, GST-catalyzed nucleophilic displacement of nitro groups (nitrite release) by glutathione has been shown for several substrates, e.g., nitroglycerin (*79*), various nitroalkanes and 4-nitropyridine-*N*-oxide (*80*). We are unaware of other literature citations that suggest a role for plant GSTs in nitro group removal from other xenobiotics.

When PCNB was metabolized in cotton (*Gossypium hirsutum* L.), pentachloroaniline (PCA) and pentachlorothioanisole (PCTA) were the major metabolites (*81*). In peanut, PCNB was rapidly adsorbed by roots, but it and its metabolites were not translocated to stem and leaf tissues (*77*). The water soluble metabolites, *S*-(tetrachloronitrophenyl)-glutathione and S-(pentachlorophenyl)-glutathione were shown to be the major initial metabolites, but in pea (*Pisum sativum* L.), *S*-(tetrachloronitrophenyl)-glutathione was only a minor metabolite (*77*). In hydroponically-grown peanut, about 60% of the radioactivity from ^{14}C-PCNB was recovered in the chloroform-extractable fraction of root tissue, with 22% identified as PCA (major metabolite) and 29 % as PCNB, 4 d after treatment (*77*). The major fate of both PCA and products of glutathione conjugation was formation of insoluble products. The peanut aryl nitroreductase responsible for the nitroreduction of PCNB was isolated and partially purified (*78*). This soluble enzyme required FAD, NADPH, and anaerobic conditions for PCNB-nitroreductase activity.

The metabolism of 2,4,6-TNT has been studied in terrestrial and aquatic plants. Yellow nutsedge (*Cyperus esculentus* L.) absorbed 2,4,6-TNT and reduced the nitro groups, yielding 4-amino-4,6-DNT and to a lesser degree, 2-amino-2,6-DNT (*82*). The deaminated metabolites were observed in all tissues (leaves, roots, rhizomes and tubers). In other studies, three terrestrial plants: bush bean (*Phaseolus vulgaris* L.), wheat (*Triticum* sp.), and bromegrass (*Bromus* sp.) were evaluated for ^{14}C-TNT uptake and transformation in soil and hydroponic systems (*83*). Bush bean had the greatest uptake with about 75% of the radioactivity recovered in the roots. Less than 20% of the radioactivity was recovered as TNT in these tissues and the remainder was amino derivatives, polar derivatives, or unextractable components. No mineralization of TNT to $^{14}CO_2$ was observed in these plant species.

Hybrid poplar trees (*Populus* sp. *Deltoides* x *nigra*) have high potential for phytoremediation of soil and water contaminated with a wide array of pesticides, explosives and other industrial chemicals (*84*). Recent studies, indicate that TNT is readily taken up and transformed by hybrid poplar seedlings (*85*). Hydroponic

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch011

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch011

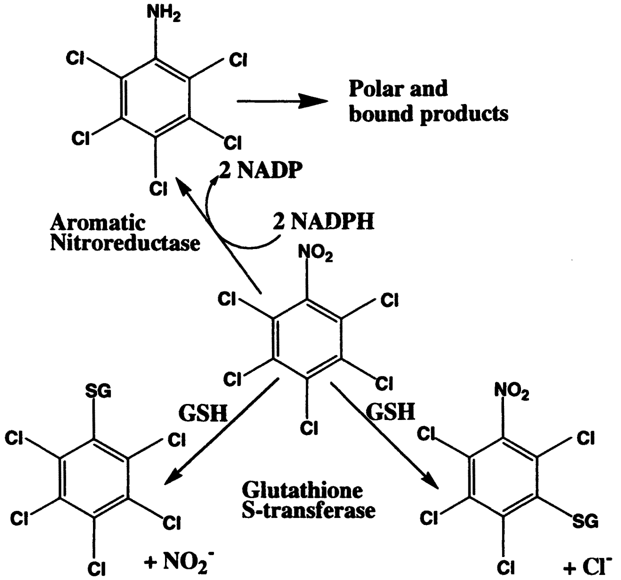


Figure 5. Transformations of pentachloronitrophenol in peanut via aromatic nitroreduction or glutathione S-transferase mediated dechlorination or nitrite release.

cultures removed about 75% of the total TNT, while about 15 and 4% of the total ^{14}C-TNT was taken up from freshly treated and aged soil, respectively. In poplars grown in all three media, most of the radioactivity was recovered in roots, and no mineralization of TNT occurred. The major TNT metabolites in poplar root tissues were 4-amino-2,6-DNT, with lesser amounts of 2-amino-4,6-DNT, 2,4-diamino-mononitrotoluene, and polar metabolites. In stems and leaves, unknown polar metabolites were the major products. The mechanism of 2,4,6-TNT nitroreduction has not been identified in any of the above plant species.

The ability of aquatic plants to transform 2,4,6-TNT has been reported. 2,4,6-TNT nitroreductase activity occurs in algae (*Nitella* sp. and *Spirogyra* sp.), and higher aquatic plants [*Myriophyllum spicatum* (Eurasian watermilfoil) and *Lemna minor* (duckweed)] (*84*). Two aquatic plant species [*M. aquaticum* (parrotfeather) and Eurasian watermilfoil] exhibited rapid uptake and metabolism of ^{14}C-TNT (*86*). Low concentrations of deaminated nitrotoluenes (2-amino-4,6-DNT and 4-amino-2,6-DNT) were observed in plant tissue and in the media. In these studies, no evidence for oxidative metabolism (e.g., mineralization) has been found, and formation of conjugates of the amino moieties has been postulated as the major detoxification mechanism.

Spinach (*Spinacia oleracea* L.) leaf ferredoxin-NADP oxidoreductase, reduced nitrobenzene to phenylhydroxylamine in the presence of an NADPH reducing system (*87*). This enzyme also transformed the explosive tetryl (2,4,6-trinitrophenylmethylnitramine) by nitrite release (*88*). *Araidopsis thaliana*

NADPH:thioredoxin reductase catalyzes the redox cycling of 2,4,6-TNT, tetryl, and DNOC, liberating nitro anion radicals (*89*). The role of these enzymes in whole plant nitroaromatic metabolism has not been established.

We are interested in modifying crop tolerance to the diphenyl-ether herbicide acifluorfen by expression of a bacterial nitroreductase gene from certain *P. fluorescens* strains. Tolerance to acifluorfen is based upon the ability of the resistant species to detoxify the herbicide via GST-mediated glutathione or homoglutathione conjugation (*90*), cleaving the diphenyl ether bond. Certain strains of *P. fluorescens* possess an oxygen-insensitive nitroreductase with a high substrate affinity for acifluorfen (*39*). The product of acifluorfen nitroreduction, aminoacifluorfen, has relatively low phytotoxicity compared to acifluorfen (unpublished results) and has limited mutagenicity in the *Salmonella typhimurium* assay (*91*). A gene therapy strategy for destroying mammalian cancer cells, based upon expression of bacterial (*E. coli*) nitroreductase (*ntr*), has been proposed (*92*). The proto-drug 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB1954) is bioactivated by this nitroreductase. When the *ntr* gene was expressed in mammalian cancer cells, the transformed cells were selectively killed when exposed to CB1954 (*93*). These studies demonstrate that prokaryotic *ntr* genes can be expressed in eukaryotic cells. Likewise, this approach maybe suitable for engineering resistance to nitroaromatic herbicides in crops and for developing novel plants useful in the phytoremediation of nitroaromatic contaminants.

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch011

Acknowledgements

This research was funded by an OECD fellowship to RMZ. HL and JTT gratefully acknowledge the financial support of NSERC (Canada) research grants.

References

1. *Biodegradation of Nnitroaromatic Compounds;* Spain, J.C., Ed.;. Environmental Sciences Research; Plenum Press: New York, NY, 1995; Vol. *49,* 232 pp.
2. Cain, R.B. *J. Gen Microbiol.* **1966,** *42,* 219-235.
3. Zeyer, J.; Kearney, P.C. *J. Agric. Food Chem.* **1984,** *32,* 238-242.
4. Delgado, A.; Wubbolts, M.G.; Abril, M.-A.; Ramos, J.L. *Appl. Environ. Microbiol.* **1992,** *58,* 415-417.
5. Hanne, L.F.; Kirk, L.L.; Appel, M.; Narayan, A.D.; Bains, K.K. *Appl. Environ. Microbiol.* **1993,** *59,* 3505-3508.
6. Gunderson, K.; Jensen, H. L. *Acta Agric. Scand.* **1956,** *6,* 100-114.
7. Zeyer, J.; Kocher, H. P. *J. Bacteriol.* **1988,** *170,* 1789-1794.
8. Spain, J.C.; Gibson, D.T. *Appl. Environ. Microbiol.* **1991,** *57,* 812-819.
9. Kadiyala, V; Spain, J.C. *Appl. Environ. Microbiol.* **1998,** *64,* 2479-2484.
10. Leung, K.T.; Tresse, O.; Errampalli, D.; Lee, H.; Trevors, J.T. *FEMS Microbiol. Letts.* **1997,** *155,* 107-144.

11. Leung, K.T.; Cassidy, M.B.; Shaw, K.W.; Lee., H.; Trevors, J. T.; Lohmeier-Vogel, E.L.; Vogel, H. J. *World J. Microbiol. Biotechnol.* **1997,** *13,* 305-313.
12. Xun, L.; Orser, C. S. *J. Bacteriol.* **1991,** *173,* 4447-4453.
13. Orser, C. S.; Lange, C. C.; Xun, L.; Zahrt, T. C.; Schneider, B. J. *J. Bacteriol.* **1994,** *175,* 411-416.
14. Xun, L.; Topp, E.; Orser, C.S. *J. Bacteriol.* **1992,** *174,* 2898-2902.
15. Raymond. D.G.M; Alexander, M. *Pestic. Biochem. Physiol.* **1971,** *1,* 123-130.
16. Jain, R.K.; Dreisbach, J.H.; Spain, J.C. *Appl. Environ. Microbiol.* **1994,** *60,* 3030-3032.
17. Leung, K. T., Campbell, S.;. Gan, Y.; Lee, H.; Trevors, J. T. UG 30. *FEMS Microbiol Letts.* **1999,** *173,* 247-253.
18. Cassidy, M.B.; Lee, H.; Trevors, J. T.; Zablotowicz, R.M. *J. Ind. Microbiol. Biotechnol.* **1999,** *23,* 232-241.
19. Zablotowicz, R.M.; Leung, K.T.; Alber, T.; Cassidy, M.B.; Trevors, J.T.; Lee, H. *Can. J. Microbiol.* **1999,** *45,* 840-848.
20. Hess, T.F.; Schmidt, S.K.; Silverstein, J.; Howe, B. *Appl. Environ. Microbiol.* **1990,** *56,* 1551-1558.
21. Lenke, H.; Pieper, D. H.; Bruhn, C.; Knackmuss, H.-J. *Appl. Environ. Micrbiol.* **1992,** *58,* 2928-2932.
22. Robertson, J.B.; Spain, J.C.; Haddock, J. D.; Gipson, D. T. *Appl. Environ. Microbiol.* **1992,** *58,* 2643-2648.
23. Ecker, S.; Widman, T.; Lenke, H.; Dickel, O.; Fischer, P.; Bruhn, C.; Knackmaus, H.-J. *Arch. Microbiol.* **1992,** *158,* 149-154.
24. Nishino, S.F.; and Spain, J.C. *Appl. Environ. Microbiol.* **1995,** *61,* 2308-2313.
25. Parales, R. E.; Ontl, T. A.; Gibson, D.T. J. *Ind. Microbiol. Bitechnol.* **1997,** *19,* 385-391.
26. Cartwright, N.J.; Cain, R.B. *Biochem. J.* **1959,** *73,* 305-314.
27. Lusby, W. R.; Oliver, J. E.; Kearney, P. C. *J. Agric. Food Chem.* **1980,** *28,* 641-644.
28. Kinouchi, T.; Yoshinari, O. *Appl. Environ. Microbiol.* **1983,** *46,* 596-604.
29. Bryant, D. W.; McCalla, D. R.; Leeksma, M.; Laneuville, P. *Can. J. Microbiol.* **1981,** *27,* 81-86.
30. Bryant, C.; Hubbard, L.; McElroy, W.D. *J. Biol. Chem.* **1991,** *266,* 4126-4130.
31. Bryant, C.; DeLuca, M. *J. Biol. Chem.* **1991,** *266,* 4119-4126.
32. Michael, N.P.; Brehm, J. K.; Anlezark, G.M.; Minton, N. P. *FEMS Microbiol. Letts.* **1994,** *124,* 195-202.
33. Yabannavar, A.V., and G.J. Zylstra. *Appl. Environ. Microbiol.* **1995,** *61,* 4284-4290.
34. Probst, G.W.; Tepe, J. B. In P.C. Kearney and D.D. Kaufman, Eds. *Degradation of Herbicides.* Marcel Dekker, Inc., NY., 1969, pp 255-282.
35. Oyamada, M.; S. Kuwatsuka, S.. *J. Pestic. Sci.* **1989,** *14,* 321-327.
36. Kulshrestha, G.; Singh, S. B. *Bull. Environ. Contam. Toxicol.* **1992,** *48,* 269-274.
37. Gennari, M.; Négre, M.; Ambosoli, R.; Andreoni, V.; Vincenti, M.; Acquati, A. *J. Agric. Food Chem.* **1994,** *42,* 1232-1236.

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch011

38. Andreoni, V.; Colombo, M.; Gennari, M.; Négre, M.; Ambosoli, R. *J. Environ. Sci. Health* **1994,** *B29,* 963-987.
39. Zablotowicz, R.M.; Locke, M. A.; Hoagland, R. E. In *Phytoremediation of Soil and Water Contaminants,* Kruger, E. L.; Anderson, T. A.; Coats, J. R. Eds., ACS Symp. Ser. 664, 1997, pp 38-53.
40. Niki, Y.; Kuwatsuka, S. *Soil Sci. Plant Nutri.* **1976,** *22,* 223-232.
41. Hamdi, Y.A.; Tewfik, M.S. *Soil Biol. Biochem.* **1970,** *2,* 163-166.
42. Golab, T.; Althaus, W.A.; Wooten, H.L. *J. Agric. Food Chem.* **1979,** *27,* 163-180.
43. Stevens, T.O.; Crawford, R.L.; Crawford, D.L. *Appl. Environ. Microbiol.* **1990,** *56,* 133-139.
44. Singh, S. B.; Kulshrestha, G. *J. Environ. Sci. Health* **1991,** *B26,* 309-321.
45. Corbett M. D.; Corbett, B. R. *Appl. Environ. Microbiol.* **1981,** *41,* 942-949.
46. Blasco, R.; Castillo, F. *Appl. Environ. Microbiol.* **1992,** *58,* 690-695.
47. Del Campo, F.F.; Ramirez, J. M.; Paneque, A.; Losada, M. *Biochem. Biophys. Res. Commun.* **1966,** *22,* 547-553.
48. Raffi, F.; Selby, A. L.; Newton, R. K.; Cerniglia, C. E. *Appl. Environ. Microbiol.* **1994,** *60,* 4263-4267.
49. Wyman, J.F.; Guard, H.E.; Won,W.D.; Quay, J.H. *Appl. Environ. Microbiol.* **1979,** *37,* 222-226.
50. Locke, M. A.; Gaston, L. A.; Zablotowicz, R.M. *J. Agric. Food Chem.* **1997,** *45,* 286-293.
51. Tewfik, M.S.; Evans, W. C. *Biochem. J.* **1966,** *99,* 31P-32P.
52. Groenewegen, P. E. J., Breeuwer, P.; van Helvoort, J. M. L. M.; Langenhoff, A. A. M.; de Vries, F. P.; de Bount, J. A. M. *J. Gen. Microbiol.* **1992,** *138,* 1599-1605.
53. Groenewegen, P. E. J.; de Bount, J. A. M. *Arch. Microbiol.* **1992,** *158,* 381-386.
54. Nishino, S.F.; and Spain, J.C. *Appl. Environ. Microbiol.* **1993,** *59,* 2520-2525.
55. Sommerville, C. C.; Nishino, S. F.; Spain, J. C. *J. Bacteriol.* **1995,** *61,* 3837-3842.
56. Fiorella, P. D.; J. C. Spain. *Appl. Environ. Microbiol.* **1997,** *63,* 2007-2011.
57. Haigler, B. E.; Spain, J. C. *Appl. Environ. Microbiol.* **1993,** *59,* 2239-2243.
58. Spiess, T.; Desiere, F.; Fischer, P.; Spain, J.C.; Knackmuss, H.-J., Lenke, H. *Appl. Environ. Microbiol.* **1998,** *64,* 446-452.
59. Schenzle, A.; Lenke, H.; Fischer, P.; Williams, P.A.; Knackmuss, H-J. *Appl. Environ. Microbiol.* **1997,** *63,* 1421-1427.
60. Schenzle, A., H. Lenke, J.C. Spain, and H-J. Knackmuss. *Appl. Environ. Microbiol.* **1998,** *65,* 2317-2323.
61. Lenke, H.; Knackmuss, H.-J. *Appl. Environ. Microbiol.* **1992,** *58,* 2933-2937.
62. Rajan, J.; Valli, K.; Perkins, R. E.; Sariaslani, F. S.; Barns, S. M.; Reysenbach, A.-L.; Rehm, S.; Ehringer, M.; Pace, N. R. *J. Ind. Microbiol.* **1996,** *16,* 319-324.
63. Behrand, C.; Heesche-Wagner, K. *Appl. Environ. Microbiol.* **1999,** *65,* 1372-1377.
64. Lenke, H.; Knackmuss, H.-J. *Appl. Environ. Microbiol.* **1996,** *62,* 784-790.

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch011

65. Vorbeck, C.; Lenke, H.; Fischer, P.; Spain, J.C.; Knackmuss, H.-J. *Appl. Environ. Microbiol.* **1998,** *64,* 246-252.
66. Hammill, T.B.; Crawford, R.L. *Appl. Environ. Microbiol.* **1996,** *62,* 1842-1846.
67. Preuβ, A.; Riegler, P.-G. In *Biodegradation of Nitroaromatic Compounds*, Spain, J.C. Ed., Plenum Press, New York, pp 69-86.
68. Crawford, R.L. In *Biodegradation of Nitroaromatic Compounds*, Spain, J.C. Ed., Plenum Press, New York, pp 87-98.
69. Scheibner, K.; Hofrichter, M.; Herre, A.; Michels, J.; Fritsche, W. *Appl. Microbiol. Biotechnol.* **1997,** *47,* 452-457.
70. Fernando, T.; Bumpus, J.A.; Aust, S.D. *Appl. Environ. Microbiol.* **1990,** *56,* 1666-1671.
71. Stahl, J.D.; Aust, S.D. *Biochem. Biophys. Res. Comm.* **1993,** *192,* 471-476.
72. Barr, D. P.; Aust, S. D. *Environ. Sci. Technol.* **1994,** *28,* 79A-87A.
73. Stahl, J.D.; Aust, S.D. *Biochem. Biophys. Res. Comm.* **1993,** *192,* 477-482.
74. Bumpus, J. A.; Tatarko, M. *Current Microbiol.* **1994,** *28,* 185-190.
75. Rieble, S.; Joshi, D. K.; Gold, M. H. *Biochem. Biophys. Res. Comm.* **1994,** *205,* 298-304.
76. Golab, T.; Herberg, R. J.; Parka, S. J.; Tepe, J.B. *J. Agric. Food Chem.* **1967,** *15,* 638-641.
77. Lamoureaux, G.L.; Rusness, D.G. *J. Agric. Food Chem.* **1980,** *28,* 1057-1070.
78. Rusness, D.G.; Lamoureaux, G.L. *J. Agric. Food Chem.* **1980,** *28,* 1070-1077.
79. Habig, W. H.; Keen, J.H.; Jakoby, W.B. *Biochem. Biophys. Res. Commun.* **1975,** *64,* 501-506.
80. Habig, W. H.; Pabst, M. J.; Jakoby, W. B. *J. Biol. Chem.* **1974,** *249,* 7130-7139.
81. Kuchar, E. J.; Geenty, F. O. Griffith, W. P.; Thomas, R. J. *J. Agric. Food Chem.* **1969,** *17,* 1237-1241.
82. Palazzo, J.; Legget, D.C. *J. Environ. Qual.* **1986,** *15,* 49-52.
83. Cataldo, D. A.; Harvey, S.; Fellows, R. J.; Bean, R. M.; McVeety, B. D. *An Evaluation of the Environmental Fate of Munitions Material (TNT, RDX) in Soil and Plant Systems.* U.S. DOE Contract 90-012748, 1989.
84. Schnoor, J. L.; Licht, L. A.; McCutcheon, S. C.; Wolfe, N. L.; Carreira, L. H. *Environ. Sci. Technol.* **1995,** *29,* 318A-323A.
85. Thompson, P. L.; Ramer L. A.; Schnoor, J. L. *Environ. Sci. Technol.* **1998,** *32,* 975-980.
86. Hughes, J. B.; Shanks, J.; Vandeford, M.; Lauritzen, J. Bhadra, R. *Environ. Sci. Technol.* **1997,** *31,* 266-271.
87. Shah, M. M.; Campbell, J. A. *Biochem. Biophys. Res. Comm.* **1997,** *29,* 794-796.
88. Shah, M. M.; Spain, J. C. *Biochem. Biophys. Res. Comm.* **1996,** *220,* 563-568.
89. Miskiniene, V; Sarlauskas, J.; Jacquot, J. P.; Cenas, N. *Biochim. Biophys. Acta* **1998,** *1366,* 275-283.
90. Frear, D.S.; Swanson, H. R.; Mansager, E. R. *Pestic. Biochem. Physiol.* **1983,** *20,* 299-310.
91. Draper, W.M.; Casida, *J. Agric. Food Chem.* **1983,** *31,* 1201-1207.
92. Anlezark, G. M.; Melton, R. G.; Sherwood, R. F.; Coles, B.; Friedlos, F.; Knox, R. *Biochem. Pharmacol.* **1992,** *44,* 2289-2295.
93. Bridgewater, J. A.; Springer, C. J.; Knox, R. J.; Minton, N. P.; Collins, M. K. *Eur. J. Cancer* **1995,** *31A,* 2362-2370.

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch011

Chapter 12

Functions and Regulation of Plant Glutathione *S*-Transferases

K. K. Hatzios

**Department of Plant Pathology, Physiology and Weed Science,
Virginia Polytechnic Institute and State University,
Blacksburg, VA 24061–0331**

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch012

Glutathione S-transferases (GSTs) are dimeric multifunctional enzymes catalyzing the nucleophilic attack of the thiol group of glutathione forming conjugates with various electrophilic substrates. GSTs are present in all plant tissues and at all stages of plant development from early embryogenesis to senescence. An established role of plant GSTs is their involvement in herbicide detoxification. Plant GSTs have also been implicated in defense mechanisms against other stress responses such as pathogenic attack, oxidative stress, heavy-metal toxicity and the cellular response to auxins and the normal metabolism of secondary plant products such as anthocyanins and cinnamic acid. Plant GSTs are characterized and differentiated by broad and partial overlapping substrate specificity, heterogeneity of subunit composition and amino acid sequences, and differential regulation by herbicide safeners. The safener-mediated regulation of plant GSTs appears to be a stepwise process, requiring signal transduction from putative safener receptors to other cellular sites.

Glutathione (γ-L-glutamyl-L-cysteinylglycine), found primarily in its reduced form (GSH), is the most important free thiol found in plant, animal, and bacterial cells (*1-4*). GSH and its homologues are found in high concentrations in plant cells, *i.e.*, 0.18 to 0.59 µmol/g of fresh weight (*1*). The highest levels of GSH are found in seeds and GSH levels vary with plant age (*1,3,4*). The foliar concentration of GSH is dependent on the influence of the reductive assimilation of sulfur, nitrogen and carbon in the amino acids glutamate, cysteine and glycine (*5*). These amino acid substrates are then converted to the tripeptide GSH in two sequential ATP-dependent reactions mediated by γ-glutamylcysteine synthease (EC 6.3.2.2) and glutathione synthetase (EC 6.3.2.3) (*4,5*). These two enzymes are distributed in chloroplastic and nonchloroplastic compartments (*5*). GSH is degraded to its constituent amino acids by the action of carboxypeptidases and transpeptidases, which are located predominantly in the cytoplasm and vacuole of plant cells (*6*). Homoglutathione (hGSH, γ-L-glutamyl-L-cysteinyl-β-alanine) is a GSH analog found in several legume plants (*7*), carrying out the functions of GSH in these plants.

In Pesticide Biotransformation in Plants and Microorganisms; Hall, J., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2000.

Due to the chemical reactivity of its sulfhydryl group, glutathione has multiple functions in higher plants. As an obligatory substrate for glutathione transferases, glutathione plays a key role in the detoxification of many herbicides and other xenobiotic or endogenous substances (*8-10*). Glutathione is also a precursor of the phytochelatins, *i.e.*, peptides which bind excess concentrations of heavy metals (*11,12*). In the chloroplast, glutathione is a key component of the ascorbate-glutathione cycle, which is very important for the scavenging of active oxygen species (*13*). Glutathione can also serve as a messenger in signal transduction and as an elicitor of chloroplast transcription (*14,15*). The oxidized form of glutathione (GSSG) has been shown to inhibit protein synthesis (*3*). At the whole plant level, GSH appears to be the major form of reduced sulfur that is translocated in the phloem and it also modulates root uptake of sulfate (*16*).

Glutathione *S*-transferases (GSTs, EC 2.1.5.18), also known as glutathione transferases, are dimeric, multifunctional enzymes occurring ubiquitously in aerobic organisms (*17-20*). GSTs catalyze the conjugation of GSH to endogenous or exogenous electrophilic substrates. The GS-conjugates formed have higher water-solubility and are less toxic than the parent compounds. In plants, GSH conjugates are eventually sequestered into the vacuole, where they undergo further degradation (18).

Reviews on the role of plant GSTs in herbicide detoxification and selectivity, their regulation by herbicide safeners, and the characterization of their isozymes and genes have been published regularly during the past decade (*8,9,18,21-24*). The present article will summarizes current knowledge on the functions or plant GSTs and the regulation of their expression by chemical modulators, mainly herbicide safeners.

Functions of Plant Glutathione Transferases

GSTs are present in every type of plant tissue examined and at every stage of plant development from early embryogenesis to senescence. Plant GSTs were first studied because of their ability to detoxify herbicides (*18,21*). Detoxification by GSH conjugation is a key factor contributing to the selectivity of several classes of herbicides in maize and other crop plants (*8,9,18,21-24*). The isomerization of thiadiazolidine herbicides to their isomeric triazolidine derivatives, which act as strong peroxidizing herbicides, illustrates a bioactivation function catalyzed by certain plant GSTs (*25,26*). The involvement of plant GTSs in the metabolism of cinnamic acid (*10*) and anthocyanins (*27*), strengthens the view that detoxification of herbicides in plants may be carried out by enzymes with normal endogenous roles in plant secondary metabolism (*28*).

In contrast to the well-established involvement of plant GSTs in herbicide detoxification, other physiological functions assigned to these enzymes are less understood at the present time. Plant GSTs have been implicated in the protection of plant tissues from oxidative damage generated by air pollutants (*29*), heavy metals (*30,31*), and pathogen attack (*32,33*). The deactivation of toxic 4-hydroxyalkenals and organic hydroperoxides, which are generated during oxidative stress, is catalyzed by the GSH-dependent peroxidase activity of plant GSTs (*34,35*).

A third function of plant GSTs is their role in the transport and regulation of certain metabolites. The conjugation of endogenous and xenobiotic substrates to GSH in plants is crucial for the transmembrane transport of the resulting GS-conjugates. Thus, GSH conjugation tags or targets substances such as anthocyanins

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch012

or herbicides for compartmentation into the vacuole (*18,27*). In addition to their catalytic function, some plant GSTs serve as nonenzymatic carrier proteins (ligandins) of endogenous substances such as the auxin, indole-3-acetic acid (IAA) (*36,37*). This nonenzymatic binding affords temporary storage of IAA and modulates its availability for binding to membrane receptors (*36,37*).

Classification of Plant Glutathione Transferases

Plant GSTs are mainly soluble (cytosolic) enzymes, expressed constitutively in specific tissues. Microsomal GST activity has been also described in some plants (*38,39*). Cytosolic GSTs represent 1% of the soluble plant protein, and they are dimeric proteins that have multiple isoforms or isozymes (*18-24*). Plant GSTs are active as homo- or heterodimers composed of subunits of about 25-29 kDa. The model substrate, 1-chloro-2,4-dinitrobenzene (CDNB), is used routinely for assaying plant GST activity. However, the use of specific herbicidal substrates is desirable for studying the activity, regulation, and classification of certain plant GST isozymes.

GSTs functioning in aerobic organisms are commonly classified on the basis of their amino acid identity, immunological cross-reactivity, and substrate specificity (9,18). According to this classification, mammalian GSTs are grouped into *alpha, mu, pi, sigma*, and *theta* classes. X-ray structures of all five classes have been determined and revealed a similar dimeric topology for all GSTs (*40*). Most cytosolic plant GSTs appear to be similar to the *theta* class of mammalian GSTs, which is also known as the ‘default class’ because of its wide distribution among different species (*41*). However, a further classification of plant GSTs into subclasses has been proposed by Droog et al. (*42*) on the basis of amino acid sequence identity of plant GST subunits and conservation of exon:intron placement of plant GST genes (where the gene structure is known). Three major classes of plant GSTs, known as type I, type II, and type III, were initially proposed by the phylogenetic scheme of Droog et al. (*42*).

Type I GSTs contain three exons and two introns and function as defense genes. Type I GSTs produce proteins that detoxify herbicides or that are active in response to pathogen attack, wounding, senescence, and the resulting lipid peroxidation that accompanies these processes (*18,42*). Some type I plant GSTs are induced in response to auxins and may serve a ligandin function toward IAA (*18*). Type II GSTs contain ten exons and nine introns and have been studied only in carnation (*Dianthus* spp.) as ethylene and senescence-related genes expressed in floral organs (*18,43*). Type III plant GSTs contain two exons and one intron (*18,42*). This set of homologous genes from a variety of plant species are inducible by diverse modulators including mainly auxin, but also ethylene, pathogen infection, heavy metals, and heat shock. Genes in this group are also known as the auxin-regulated gene (ARG) subgroup. Recently, Droog (*44*) and Dixon et al. (*45*) updated the phylogenetic classification system of plant GTSs into the following four classes: type I or *theta* class, type II or *zeta* class, type III or *tau* class, and type IV class.

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch012

The Glutathione Transferase Gene-Enzyme System

GST Isozymes in plants

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch012

GSTs are encoded by a multigene family producing isozymes with broad substrate specificities. In maize (*Zea mays* L.), at least eight dimeric isoforms of cytosolic GSTs have been described to date. Seven of these dimeric GSTs are involved in the metabolism of herbicidal substrates and are formed from specific combinations of five GST subunits, which have been characterized. *Bronze* 2 is an inducible type III (*tau*) maize GST (26 kDa) with activity towards CDNB and natural substrates such as anthocyanin pigments (*18*). A monomeric GST of 30 kDa, which utilizes phenylpropanoids has been also described in maize (*46*), but it is now believed to be ascorbate peroxidase rather than GST (*47*). Older published reports suggest the presence of two additional GST isoforms in maize, with activity towards the herbicides atrazine [6-chloro-*N*-ethyl-*N'*-(1-methylethyl)-1,3,5-triazine-2,4-diamine] (*48*) and EPTC-sulfoxide (sulfoxide of *S*-ethyl dipropyl carbamothioate) (*49*).

The first four GST isozymes characterized in maize were termed GST I – GST IV in order of their discovery and on the basis of their activity towards choroacetanilide herbicides and their inducibility by herbicide safeners (*18,23*). A revised nomenclature system for plant GST isozymes based on their subunit composition has been proposed recently by Dixon et al. (*45*). According to this system, maize GSTs are termed *Zm* GST, where the prefix *Zm* indicates *Zea mays* as the plant source. The system then utilizes the five GST subunits that have been characterized in maize to name the maize GSTs. The five GST subunits are named *Zm* GST I (29 kDa), *Zm* GST II (27 kDa), *Zm* GST III (26 kDa), *Zm* GST V (28.5 kDa), and *Zm* GST VI (27.5 kDa). The first three subunits belong to the type I or *theta* class of plant GSTs, whereas the other two in the type III or *tau* class (*26,44,45*). These subunits associate to form the following seven GST isozymes of maize: *Zm* GST I-I (GST I in old nomenclature); *Zm* GST I-II (old GST II); *Zm* GST III-III (old GST III); *Zm* GST II-II (old GST IV); *Zm* GST I-III; *Zm* GST V-V; and *Zm* GST V-VI (*50*).

Zm GST I-I (old GST I) is expressed constitutively in maize roots and shoots and is a homodimer of two 29 kDa (*Zm* GST I) subunits. It is moderately induced by safeners and shows activity towards alachlor [2-chloro-*N*-(2,6-diethylphenyl)-*N*-(methoxymethyl)acetamide], atrazine and CDNB (*18,51-55*). This isozyme catalyzes also the isomerization of thiadiazolidin-one herbicides to triazolidin-one-thiones, which are more active peroxidizing derivatives (*56*). *Zm* GST I-II (old GST II) is heterodimer of the 29 kDa (*Zm* GST I) and 27 kDa (*Zm* GST II) subunits, and is an inducible isozyme formed in response to treatment of maize with the safeners flurazole [phenylmethyl 2-chloro-4-(trifluoromethyl)-5-thiazolecarboxylate] and dichlormid (2,2-dichloro-*N,N*-di-2-propenylacetamide) (*18,52-55,57*). It is active towards alachlor and CDNB and catalyzes the isomerization of thiadiazolidin-one herbicides more efficiently than *Zm* GST I-I (*18,52-56*). *Zm* GST III-III (old GST III) is a homodimer of two 26 kDa (*Zm* GST III) subunits and is active towards alachlor, metolachlor [2-chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-(2-methoxy-1-methylethyl) acetamide] and CDNB (*58-60*). It is inducible by the safener dichlormid (*18*) and by cadmium (*61*). *Zm* GST II-II (old GST IV) is a homodimer of two 27 kDa (*Zm* GST II) subunits. It is inducible by the safener benoxacor [4-(dichloroacetyl)-3,4-dihydro-3-methyl-2*H*-1,4-benzoxazine] and is active towards acetochlor [2-chloro-*N*-(ethoxymethyl)-*N*-(2-ethyl-6-methylphenyl)acetamide], alachlor, and metolachlor (*53,62,63*). *Zm* GST I-III is a constitutive heterodimer of a 29 kDa (*Zm* GST I) and a 26 kDa (*Zm* GST III) subunit with activity towards chloroacetanilide herbicides and fluorodifen [2-nitro-1-(4-nitrophenoxy)-4-trifluoromethylbenzene] (*64*). *Zm* GST V-V is a homodimer of two 28.5 kDa (*Zm* GST V) subunits with activity towards diphenyl ether herbicides and is inducible by the safener dichlormid (*50*). *Zm* GST V-VI is a heterodimer of the 28.5 kDa (*Zm* GST V) and 27.5 kDa (*Zm* GST VI) subunits with activity on metolachlor and is an auxin-inducible isoform of maize (*50*). A summary of our current understanding of the GST gene-enzyme system of maize is given in Table I.

Compared to maize, little is known about GST isozymes in other plant species. Multiple GST isoforms conjugating herbicides have been identified and characterized recently in wheat (*Triticum aestivum* L.) (*24,65,66,67*). Eight dimeric GSTs of wheat, three of which are constitutive and five that are inducible by safeners, have been described by Cummins et al. (*65*). An additional GST isozyme which is inducible by the safener naphthalic anhydride (NA), has been recently characterized by Pascal and Scalla (*66,67*). The wheat GSTs are homodimers or heterodimers resulting from the association of 24 kDa, 25.5 kDa, and 26 kDa subunits (*24*). All of the described wheat GSTs show activity towards metolachlor and fluorodifen, while six of these enzymes were also active with the herbicide fenoxaprop {(±)-2-[4-[(6-chloro-2-benzoxazolyl)oxy]phenoxy]propanoic acid} (*24*). In addition, wheat GSTs were also capable of metabolizing isothiocyanate and crotonaldehyde (*24*). This suggests that these enzymes may also participate in the protection of wheat against oxidative damage (*24*). A pathogen-induced wheat GST, with high activity towards CDNB has been described by Mauch and Dubler (68,*69*). Two other wheat GSTs, GST25 and GST26, are strongly induced by cadmium and herbicides such as atrazine, alachlor, and paraquat (1,1'-dimethyl-4,4'-bipyridinium ion), but not by pathogen attack (*32*).

Constitutive and safener-inducible GST isozymes have been also characterized in grain sorghum [*Sorghum bicolor* (L.) Moench.] by Gronwald and Plaisance (*70*). GST A1/A1 is a constitutively expressed homodimer of two 26 kDa subunits, exhibiting high activity with CDNB, but low activity towards metolachlor (*70*). GST B1/B2 is a heterodimer composed of a 26 kDa subunit (designated the B1 subunit) and a 28 kDa subunit (designated as the B2 subunit) (*70*). This GST isozyme of grain sorghum exhibited low activity towards CDNB and high activity with metolachlor (*70*). Both sorghum GSTs appear to be glycoproteins because of their ability to bind to concanavalin A (*70*). The amino acid sequences of the GST A1, B1, and B2 subunits showed high homology with the maize *Zm* GST I (29 kDa) subunit and were classified as members of the *theta* class of plant GSTs (*70*).

Wu et al. (70-75) proposed that rice (*Oryza sativa* L.) contains at least three GST isozymes. Two of them, designated as RGST I (*Os* GST I) and RGST II (*Os* GST II), appear to be constitutively expressed in rice roots and their activity was induced by the safener fenclorim (4,6-dichloro-2-phenyl-pyrimidine) (*72-74*). Phylogenetic analysis showed that *Os* GST I appeared to be more closely related to the wheat GSTs, while *Os* GST II was more closely related to the maize *Zm* GST I-I and *Zm* GST II-II isozymes (*72*). An additional rice GST isozyme, involved in cold tolerance and belonging to class III (or *tau*), has been described by Binh and Oono (*76*).

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch012

Table I. The Glutathione Transferase Gene-Enzyme System of Maize

Gene	*Subunit*	*Isozyme*	*Substrate*	*Expression*
Gst29	*Zm* GST I (29 kDa)	*Zm* GST I-I (homodimer)	Acetanilides, CDNB, Atrazine Thiadiazolidinones	Constitutive
Gst27	*Zm* GST II (27 kDa)	*Zm* GST II-II (homodimer)	Acetanilides	Safener-inducible
Gst29 *Gst27*	*Zm* GST I *Zm* GST II	*Zm* GST I-II (heterodimer)	Acetanilides CDNB Thiadiazolidinones	Constitutive (roots) Safener-inducible
Gst26	*Zm* GST III (26 kDa)	*Zm* GST III-III (homodimer)	Acetanilides CDNB	Constitutive Inducible by Cd
Gst29 *Gst26*	*Zm* GST I *Zm* GST III	*Zm* GST I-III (heterodimer)	Acetanilides Fluorodifen	Constitutive
na	*Zm* GST V (28.5 kDa)	*Zm* GST V-V (homodimer)	Diphenyl ethers	Safener-inducible
na	*Zm* GST V *Zm*GST VI (27.5 kDa)	*Zm* V-VI (heterodimer)	Acetanilides	Auxin-inducible
Bz2	*Zm* Bz2 (26 kDa)	*Zm* BZ2 (homodimer)	Cyanidin-3-glucoside CDNB	Inducible by Cd, Abscisic acid, arsenite

NOTE: na = not available
SOURCE: Compiled from information reviewed in references (*18,23,24,26,50,85*).

Two GST isozymes active towards the herbicide fluorodifen have been described in pea (*Pisum sativum* L.) (*24,35*). One of them appeared to be a homodimer of two 30 kDa subunits, while the other is a heterodimer composed of a 30 kDa and a 27.5 kDa or 29 kDa subunits. High GST activities towards the diphenyl ether herbicide fluorodifen have been found also in other legume crops such as *Phaseolus* sp. and white clover (*Trifolium repens* L.) (*24,35*). The enzymes responsible for the homoglutathione conjugation of metribuzin sulfoxide [sulfoxide of 4-amino-6-(1,1-dimethylethyl)-3-(methylthio)-1,2,4-triazin-5(4*H*)-one] and chlorimuron ethyl {2-[[[[(4-chloro-6-methoxy-2-pyrimidinyl)amino]carbonyl]amino]sulfonyl]benzoic acid}

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch012

in soybean [*Glycine max* (L.) Merr.] have not been isolated or characterized. However, Flury et al. (*77*) have purified an inducible dimeric GST from soybean composed of two 26 kDa subunits, which is active towards metolachlor but not fluorodifen or atrazine. Furthermore, soybeans have at least three additional GSTs, which were originally described as heat shock or auxin-inducible proteins (*28*). GST1 (also known as GH2/4 or *Gm* phsp26-A) was originally cloned as a heat shock protein and an auxin-induced protein (*78,79*). On the basis of its homology to other cloned GSTs and its activity toward CDNB, this protein was later identified as a GST (*80*). Two other GST proteins from soybean embryos, one with homology to GH2/4, and one with homology to the *Zm* GST I (29 kDa) subunit of maize have been also described (*28*). Lastly, GST isozymes capable of conjugating herbicides have been purified and characterized from the weeds giant foxtail (*Setaria faberi* Hermm.) (*81*) and velvetleaf (*Abutilon theophrasti* Medik.) (*82-84*).

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch012

GST Genes in Plants

The number of characterized plant GST protein or gene sequences is growing rapidly. As of today, more than 50 partially or fully sequenced genes from 13 plant species, coding GSTs have been published (*18,44,45,72*). The list includes *Arabidopsis* with ten cDNA or genomic GST sequences, tobacco (*Nicotiana tabacum* L.) with nine, maize with seven, soybean with six, carnation (*Dianthus caryophyllus* L.) with three, rice with four, wheat with three, and mung bean [*Vigna radiata* (L.) Wilczek], potato (*Solanum tuberosum* L.), curled leaved tobacco, broccoli (*Brassica oleracea* L.), *Silene cucubalus*, *Hyoscyamus muticus*, and blue gum (*Eucalyptus globulus* Labill), each with one (*18,44,45,72*).

GST nucleotide sequences are available for cDNA and/or genomic clones encoding four of the five known GST subunits of maize. Thus, the genes *Gst29*, *Gst27*, and *Gst26* encode the *Zm* GST I (29 kDa), *Zm* GST II (27 kDa), and *Zm* GST III (26 kDa) subunits of maize GSTs (18). A cDNA clone coding for the *Zm* GST V (28.5 kDa) subunit of maize has been also described (*50*). Genetic and biochemical studies reported by Frova et al. (*85*) showed that the maize *Gst27* gene, coding for the *Zm* GST II subunit of the *Zm* GST II-II isozyme, plays a major role in the tolerance of maize genotypes to the acetanilide herbicide alachlor. Although the isozymes *Zm* GST I-I and *Zm* GST I-II appeared to be required to provide complete alachlor tolerance in maize genotypes, the lack of expression of *Zm* GST II-II was responsible for the susceptibility or moderate tolerance of several maize genotypes (*85*). Further proof supporting this conclusion was provided by studies of the expression of maize GST genes in transgenic tobacco plants. Jepson et al. (*86*) were able to express high levels of the maize *Gst29* and *Gst27* genes in transgenic tobacco, but only lines transformed with the *Gst27* gene were tolerant to chloroacetanilide herbicides. Apparently, *Zm* GST II subunits coded by the maize *Gst27* gene were capable of forming active forms of the homodimeric isozyme *Zm* GST II-II, which afforded herbicide resistance to the transgenic tobacco lines (*86*). Sommer and Böger (26) recently reported that maize GST isozymes, including the 27 kDa subunit (*Zm* GST II), also show high glutathione peroxidase activity utilizing three different organic hydroperoxides as substrates. Further details on the peroxidase activity of maize GSTs are presented by these authors in another chapter of this volume. Apart from the maize genes coding for herbicide-detoxifying GSTs, the *Bronze-2* gene of maize (*Zm Bz*-2), which encodes for a GST facilitating the vacuolar transport of endogenous anthocyanins, is also well characterized (*87*).

A synthetic gene encoding a maize GST with activity towards the herbicide atrazine was constructed in 1989 by Wosnick et al (*88*). This GST gene was introduced into *Escherichia coli* and was successfully expreseed (*88*). The introduction of this gene into sensitive plants to confer tolerance to triazine herbicides was mentioned by Wosnick et al. (*88*), but no follow-up reports have been published. In any case, the development of transgenic tobacco plants containing a gene encoding a GST detoxifying atrazine by scientists at CIBA-Geigy (also Novartis, and now Syngenta) was reported by Chilton (*89*). Atrazine-tolerant lines of tobacco were tested by CIBA-Geigy in North Carolina in the summer of 1987 (*89*). However, the planned development of atrazine-resistant lines of soybean by CIBA-Geigy was not implemented because of the successful commercialization of the sulfonylurea herbicides by DuPont, which were selective for soybeans.

A pathogen-induced wheat gene, *gstA1*, coding for a GST possessing strong activity for CDNB has been described by Mauch and Dudler (*69*). Two other GST genes coding for the wheat GST25 and GST26 isozymes were induced by herbicide treatment, but not by pathogen attack (*32*). Two cDNA clones (named *Rgst I* and *Rgst II*) encoding two putative GST isozymes in rice have been isolated from rice roots by Wu et al. *(72-74*). Northern blotting with the two clones as probes showed that the GST genes are constitutively expressed in roots of unsafened rice seedlings. Treatment with the safener fenclorim, induced the expression of the rice genes and the coded GST isozymes. The isolation of a full-length cDNA encoding the second GST isozyme of rice has been also reported by Wu et al. (*75*).

Soybeans have at least four different GST genes, which exhibit a range of environmental and developmental responses, and were originally described as genes coding for heat shock or auxin-inducible proteins (*28*). GST1 (formerly known as GH2/4 or*Gm* phsp26-A) was originally cloned as a heat-shock and an auxin-induced gene (*78.79*). On the basis of its homology to other cloned GSTs, this gene was later identified as a GST gene of class III (*80*). *GSTa*, a gene coding for another soybean GST, has been shown to be an 2,4-D-inducible gene (*77*), sharing 37.4% overall homology with GH2/4 at the gene level (*77,90*). Two other GST genes have been cloned from soybean embryos: one with homology to GH2/4, and one with homology to the *Zm* GST I (29 kDa) subunit of maize (*28*). More recently, Andrews et al. (91) reported the characterization of two cDNAs, which code for the *Gm*GST2 and *Gm*GST3 isozymes of soybeans.

Role of Glutathione Conjugation in Herbicide Metabolism

Most herbicides are metabolized by a well-defined group of enzymes that catalyze various oxidation, hydrolysis, and conjugation reactions (*92-97)*. The enzymatic detoxification of herbicides in plants is a stepwise process starting with oxidative and hydrolytic reactions, that predisposes the parent molecule to subsequent conjugation with an endogenous substance such as glutathione, glucose, or an amino acid. The formed herbicide conjugates are susceptible to further processing by means of secondary conjugation, catabolism and compartmentation. The capacity of plants to

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch012

detoxify certain herbicides by these enzymatic reactions is not uniformly distributed among various plant species. This factor has long been recognized as an important process contributing to the selectivity of herbicides (*92-95*).

The detoxification of herbicides and other xenobiotics by glutathione conjugation depends greatly upon the levels of glutathione and the activity of specific GST enzymes found in plant species. For example, the tolerance of sixteen plant species to chloroacetanilide herbicides has been positively correlated with GSH or hGSH levels, and with the rate of GSH or hGSH conjugation (*96*). When GSH and GST levels were induced with dichloroacetamide safeners, plant resistance to these herbicides increased (*97*). On the other hand, when GSH in maize seedlings was depleted with buthionine-*S,R*-sulfoxamine, an inhibitor of γ-glutamylcysteine synthetase, the phytotoxicity of metolachlor increased (*98*).

In addition to chloroacetanilides, other herbicides known to conjugate with glutathione include sulfoxidized thiocarbamates (*e.g.*, EPTC), chloro-*s*-triazines (*e.g.*, atrazine), triazinone sulfoxides (*e.g.*, metribuzin), sulfonylureas (*e.g.*, chlorimuron; triflusulfuron, {2-[[[[4-(dimethylamino)-6-(2,2,2-trifluoroethoxy)-1,3,5-triazin-2-yl]amino]carbonyl]amino]sulfonyl]-3-methylbenzoic acid}; thifensulfuron {3-[[[[4-methoxy-6methyl-1,3,5-triazin-2-yl)amino]carbonyl]amino]sulfonyl]-2-thiophene-carboxylic acid}), aryloxyphenoxypropionates (*e.g.*, fenoxaprop-ethyl), diphenylethers (*e.g.*, flurodifen; acifluorfen), oxyacetamides (*e.g.*, fluthiamide {N-(4-fluorophenyl)-N-(1-methylethyl)-2-[[5-trifluoromethyl)-1,3,4-thiadiazol-2-yl]oxy]-acetamide}), thiodiazolidines, and sulfonamides (*e.g.*, cloransulam-methyl {3-chloro-2-[[5-ethoxy-7fluoro[1,2,4-]triazolo[1,5-c]pyrimidin-2-yl)sulfonyl]amino]benzoic acid, methyl ester}) (*24*). The chemical structures of some herbicides that are known substrates of plant GSTs are shown in Figure 1.

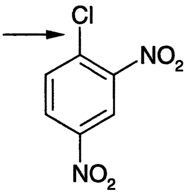
The discovery of 10-fold atrazine resistance, developed in two biotypes of velvetleaf from Maryland and Wisconsin, has been shown to result from an enhanced detoxification of atrazine via glutathione conjugation (*82,83*). The mechanism of velvetleaf resistance to atrazine appears to involve an enhanced catalytic constant for GST activity utilizing atrazine as substrate (*84*).

Catalytic Mechanisms of Plant GSTs Detoxifying Herbicides

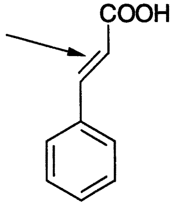
GSTs catalyze the nucleophilic attack of the sulfur atom of glutathione (GSH) to the electrophilic center of various herbicides or endogenous compounds. The recent elucidation of crystal structures of three plant GSTs (one from *Arabidopsis* and two from maize) has advanced our knowledge on the interactions of the active site of GSTs and their substrates at the molecular level (*99-101*). X-ray crystallography demonstrated that the three plant GSTs analyzed are homodimers, and that each subunit consists of two spatially distinct domains connected by a linker segment of variable length (*9,99-101*). The smaller *N*-terminal domain (domain I) is highly conserved and specific amino acid residues facilitate the binding of GSH or maintain enzyme structure. For example, a characteristic *cis*-proline bond observed in the three plant GST structures (*9,99-101*), is crucial for the correct folding of the glutathione binding site. In addition, the *N*-terminus is responsible for the catalytic activity through activation of the GSH thiol group in four classes of GSTs. The larger *C*-

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch012

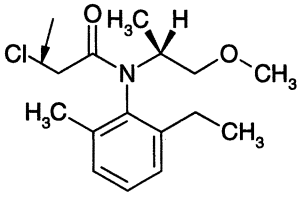
Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch012



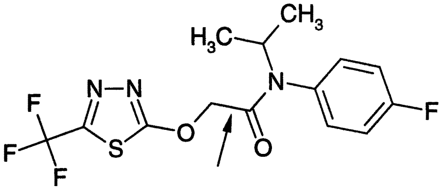
CDNB



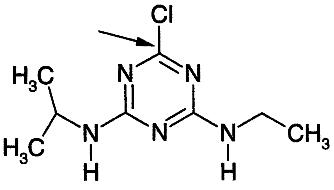
trans-cinnamic acid



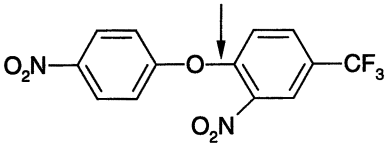
Metolachlor



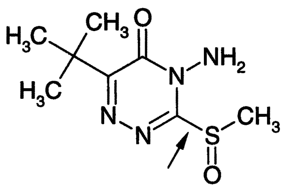
Fluthiamide (BAY FOE 5043)



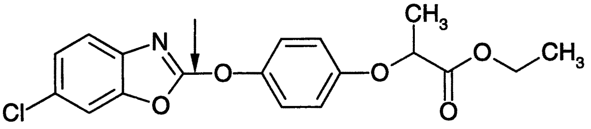
Atrazine



Fluorodifen



Metribuzin Sulfoxide



Fenoxaprop-ethyl

Figure 1. *Chemical structures of herbicides known to conjugate with GSH in plants. Arrows show sites of glutathione attack.*

terminal domain (domain II) is less conserved, completely α-helical, and consists of six amphipathic helices forming a right-handed spiral (*9,99-101*).

Each subunit contains a kinetically independent active site, composed of two distinct subsites known as the G-site and H-site. The hydrophilic and highly specific G-site is the glutathione-binding site, while the adjacent hydrophobic H-site is less specific and promotes the binding of structurally diverse substrates (*9,18*). The structural homology shared by plant and mammalian GSTs is significant, but they differ in the *C*-terminal region, where plants seem to have a broader and deeper H-site (*9,18*). In general, amino acid residues present in the G-site are conserved or conservatively replaced, whereas variations within the H-site are more common, constituting an important part of the structural diversity of GSTs (*9,18*). A molecular model for the binding of the glutathione conjugate of the herbicide fluthiamide to the active site a GST of *Arabidopsis thaliana* was published recently by Bieseler et al. (*102*).

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch012

Regulation of Glutathione-Mediated Reactions

Enzymes involved in the synthesis of 'defense' compounds (many secondary plant metabolites), may be triggered by several inducers such as mechanical wounding, infection and stress conditions including chemical treatment (*94,95*). The inducibility of the enzymatic systems detoxifying xenobiotics in plants and animals by the same biotic and abiotic factors indicates that regulation mechanisms may have been conserved along with the catalytic function of these enzymes during evolution (*28*).

The chemical regulation of metabolism-based tolerance by herbicide synergists and safeners is conceptually simple. If tolerance is due to a single detoxification reaction, herbicidal activity can be enhanced with inhibitors or decreased with enhancers of the detoxifying enzyme. Indeed, most safeners and synergists seem to act as 'bioregulators' that modulate the amount of active herbicide that reaches its target site (*103-105*). This is by far the most widely understood and common mechanism of safening or synergizing herbicides used in modern crop production.

Safeners and Glutathione-mediated Reactions

Safeners (also known as antidotes) are chemical agents that reduce the phytotoxicity of herbicides to crop plants by a physiological or molecular mechanism (*103-105*). At present, most commercialized safeners are chemical compounds that structurally resemble the herbicides that they antagonize on selected crops (*103-105*).

Grass crops protected by herbicide safeners are moderately tolerant to the antagonized herbicides, and all safeners are most effective when applied prior to or simultaneously with their respective herbicides. In practice, safeners are applied either to the crop seed prior to planting (*seed safeners*) or to the soil or crop together with the herbicides in a single formulation package (*103,104*). Seed safeners include mainly the safeners used with grain sorghum and the safener NA (naphthalic anhydride), which can be used with maize or other crops. With the exception of NA, all maize safeners contain a dichloromethyl group, are effective against thiocarbamates and chloroacetanilides, and are applied as prepackaged formulated mixtures with the respective herbicide. Safeners and their effects on herbicide action and metabolism is the subject of another chapter by Ramsey et al. in this volume.

Safeners enhance the glutathione conjugation of chloroacetanilide and sulfoxidized thiocarbamate herbicides either by elevating the levels of reduced glutathione (GSH) or by inducing the activity of glutathione-dependent enzymes. Safeners may elevate GSH levels in protected plants either directly or indirectly by: a) regulating the assimilatory sulfate reduction to cysteine; b) activating key enzymes involved in the biosynthesis of GSH; and c) inducing the activity of glutathione reductase (*103,105*).

Enhanced metabolism of chloroacetanilide and sulfoxidized thiocarbamate herbicides by GSH conjugation could also result from a safener-induced increase of the activity of the respective GST enzymes which catalyze this reaction in protected grass crops. A strong correlation between the ability of a safener to increase GST activity, and its efficacy in protecting maize, grain sorghum, rice and wheat from chloroacetanilide injury, has been demonstrated (*103,105,106*). Pretreatment of maize, grain sorghum and rice with safeners such as dichlormid, benoxacor, flurazole and fenclorim greatly enhances their low intrinsic tolerance to thiocarbamate and chloroacetanilide herbicides by inducing GST activity, and in turn elevating the rate of the herbicide detoxification via GSH conjugation. The safeners themselves are not toxic, but do exhibit structural similarities to herbicides and appear to act by inducing gene expression. The exact mechanism of the safener-mediated enhancement of GST activity is not completely understood, but appears to be a stepwise process, requiring signal transduction from putative safener receptors to other cellular sites, which results in the transcriptional activation of specific plant GSTs (*106*). In addition to the aforementioned safeners, NA and benzenesulfonamide safeners are also known to induce the expression of genes coding for GSTs and other enzymes in maize (*107*), *Arabidopsis thaliana* (*108*) and wheat (*69*). The chemical structures of herbicide safeners known to enhance GST activity are shown in Figure 2.

Synergists and Glutathione-mediated Reactions

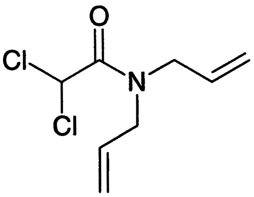
Herbicides detoxified by GSH conjugation can be synergized by the manipulation of GST activity or GSH levels. Large-seeded crop plants such as maize and soybean tend to have higher levels of GSH or hGSH in the very early seedling stage than small-seeded weeds, so synergism by depletion of GSH or GST is a possible method of herbicide synergism (*109*).

A successful and practical example of this approach is the use of the herbicide tridiphane [2-(3,5-dichlorophenyl)-2-(2,2,2-trichloroethyl)oxirane] (Figure 3) as a selective synergist of atrazine on several panicoid grass weeds such as fall panicum (*Panicum dichotomiflorum* Michx.), giant foxtail, proso millet (*Setaria italica* (L.) Beauv.), and large crabgrass (*Digitaria sanguinalis* (L.) Scop.) (*110-112*). The basis for this synergistic interaction is inhibition of atrazine detoxification by tridiphane. Boydston and Slife (*110*) showed that tridiphane inhibited the metabolism of atrazine in both maize and giant foxtail, but maize was still able to metabolize the majority of atrazine in the presence of tridiphane.

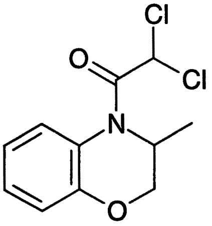
Atrazine is metabolized by GSH conjugation in both maize and giant foxtail and the selective synergism of atrazine by tridiphane in giant foxtail appears to be due to

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch012

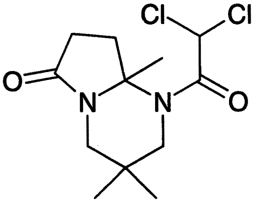
Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch012



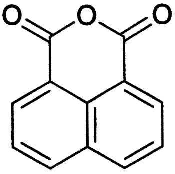
Dichlormid



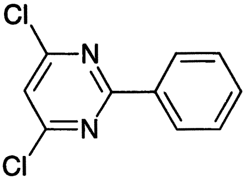
Benoxacor



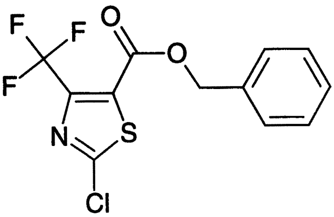
BAS-145138



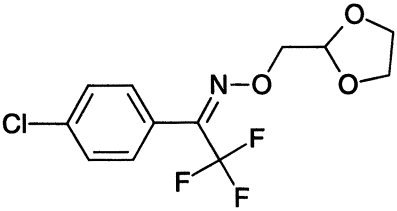
Naphthalic anhydride



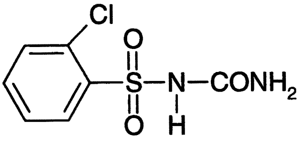
Fenclorim



Flurazole



Fluxofenim



Benzenesulfonamide

Figure 2. *Chemical structures of herbicide safeners known to induce GST activity in plants.*

differences in the inhibition of the GST-catalyzed conjugation of atrazine (*110,111,113*). Although tridiphane mainly synergizes atrazine, synergism has also been reported for combinations of tridiphane with EPTC or alachlor in maize and fall panicum (*109*) and with cyanazine in giant foxtail (*110*). Tridiphane inhibited the *in vitro* GSH conjugation of PCNB (pentachloronitrobenzene), fluorodifen, and propachlor [2-chloro-*N*-(1-methylethyl)-*N*-phenylacetamide] in extracts from pea and equine liver (*114*). In addition, tridiphane acts as a synergist of diazinon[*O,O*-diethyl *O*-(2-isopropyl-6-methyl-4-pyrimidinyl) phosphorothioate] in the resistant housefly by inhibiting GST activity (*111,115*).

In plants, tridiphane is converted to its tridiphane-glutathione conjugate (Figure 3), which appears to be the actual GST inhibitor (*111*). The tridiphane-GS conjugate is stable in giant foxtail, but unstable in maize. The tridiphane-GS conjugate inhibits a variety of GST enzymes with several different substrates, and has been reported to be a competitive inhibitor with respect to GSH (*111*).

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch012

Regulation of GST Gene Expression

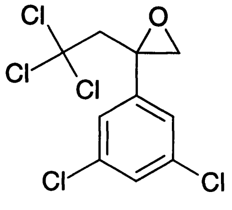
The expression of genes coding for GSTs in plants may be regulated by wounding, fungal pathogens, ethylene, heat shock, heavy metals, auxins, and herbicide safeners (*18,44*). Because of the broad nature of these modulators, it seems likely that the safener-mediated induction of herbicide degrading enzymes may be a part of a general stress response.

In contrast to mammalian systems, where Xenobiotic Regulating Elements (XRE) are found in multiple copies of GST and P-450 genes (*19*), plant GST promoters do not contain functional XREs or Electrophile Responsive Elements (EpREs) (*18*). Instead, some of the aforementioned inducers of plant GSTs seem to work through a single element in the promoter, termed the *ocs* (octopine synthase) element (*116*). The *ocs* elements are 20-bp in size, identified first in promoters of genes from plant pathogens (CaMV and *Agrobacterium tumefaciens*), and are activated by wounding (*116*). The *ocs* elements of plant GST promoters appear to be stress-inducible elements similar to the Activator Protein-1 (AP-1) sites and respond to biotic and abiotic agents generating conditions of oxidative stress (*18,116*). However, it must be noted that so far, *ocs* elements have only been found in promoters of GST genes belonging to the type III or *tau* class (*44*).

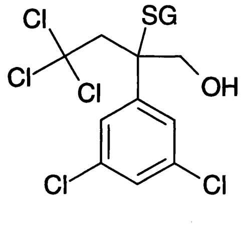
It is expected that multiple regulatory elements are present in the promoters of most GST genes, some of which will react to specific signals, and some of which to more general stress-related signals (*44*). While genes of the *theta* class of plant GSTs have not been shown to contain *ocs* elements in their promoters, they are induced at the mRNA level by herbicide and safener treatments (*18*). A time-course of the induction of mRNA expression of the *RgstII* gene of rice by the safener fenclorim is shown in Figure 4. The existence of a Safener Response Element (SRE) in the promoters of maize GSTs, consisting of the sequence –ATTTCAAA-, has been proposed recently by Jepson et al. (*117*).

The involvement of a common factor in the signaling transduction pathway, from the initial recognition of the stimulus to the activation of gene expression of plant *gst* genes, is very likely. This is supported by the fact that a subset of *gst* genes are activated by oxidative stress and the activity of the encoded proteins is needed for the

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch012



Tridiphane



Tridiphane-GS conjugate

Figure 3. Chemical structures of the herbicide synergist tridiphane and of the GS-tridiphane conjugate.

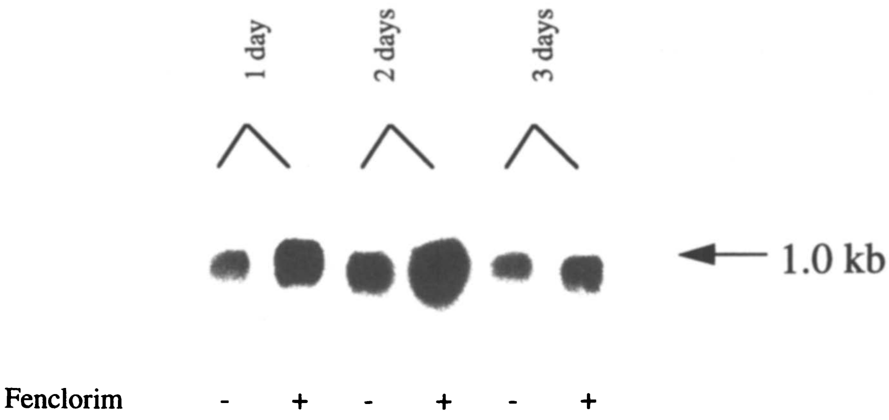


Figure 4*. Northern blot analysis of total RNA isolated from roots of rice seedlings at 1, 2 and 3 days after safener treatment and probed with ^{32}P-radiolabeled Rgst II cDNA. Within each time period, the first lane corresponds to untreated (control) rice roots and the second lane to fenclorim-treated (1 μM) rice roots. (Reproduced with permission from reference 72).*

cellular protection against oxidative damage (*118,119*). Treatment of plants with chemicals such as herbicides and safeners constitutes a type of stress, and will therefore elicit a signal over one of the different stress signaling chains present in plants (e.g., jasmonic acid and other plant hormones).

Stress treatments induce an oxidative burst like the one studied mostly in pathogen infections (*118,119*). The active oxygen species (AOS) generated either directly or indirectly in plant-pathogen systems, lead to the activation of defense genes including GSTs, that are needed to protect the cells against oxidative damage (*18*). In addition, AOS stimulate phyotalexin biosynthesis and promote the hypersensitive response of pathogen-infected tissues of plants. AOS lead to membrane damage and the generation of hydroperoxides, which might be the actual inducers of GST genes in plants (*18,44*).

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch012

Fate of Glutathione Conjugates in Plants

Glutathione conjugates of herbicides in plants are catabolized to their cysteine and thiolactic derivatives, which are further acylated with malonic acid (*120*). These terminal metabolites may be stored as soluble metabolites in plant cell vacuoles, or deposited as bound residues in biopolymers found in the plant cell walls (*120,121,122)*. Further details on the vacuolar compartmentation of detoxified herbicides and other xenobiotics can be found in recent reviews by Blake-Kalff *et al.* (*122*), Sandermann *et al.* (*121*) and Schröder (*120*). Studies on the potential effects of safeners on the formation of secondary conjugates of herbicide metabolites are scarce. The dichloroacetamide safener BAS-145138 {1-dichloroacetyl-hexahydro-3,3-8α-trimethyl-pyrrolo-[1,2-α]-pyrimidin-6-(2H)-one} caused no significant alterations in the formation of soluble secondary metabolites and bound residues from the initial glutathione conjugates of propachlor and metolachlor in maize (*123*).

Glutathione pumps (also known as GS-X pumps) are present in the plant tonoplast membrane. The vacuole appears to serve as a place for the temporary storage of glutathione conjugates of xenobiotic and endogenous substances (*122,124-128*). Plant GS-X pumps contain a region that undergoes phosphorylation (P-domain), a site that recognizes GSH (G-domain), and a site with affinity toward the electrophilic moiety of the glutathione conjugates (C-domain) (*18,125-128*). Vacuolar peptidases can cleave the glutathionyl-moiety of the conjugates (*120*). The resulting cysteinyl conjugates may reappear in the cytosol or the apoplast as stable end-products or as reactive intermediates available for further metabolism (*120*).

The vacuolar transport of glutathione conjugates of specific herbicides and other xenobiotics is affected by some herbicide safeners (*125-128*). Glutathione conjugates of CDNB and chloroacetanilide herbicides are compartmentalized in cell vacuoles by a membrane transporter that is energized by MgATP (*126-128*). Pretreatment with CDNB increases the amount of recruitment of functional transporter into the vacuolar membrane (*127*). The safener benoxacor antagonized the effects of CDNB on this system (127). Gaillard *et al.* (*128*) showed that apart from inducing the activity of herbicide detoxifying enzymes, the safener cloquintocet-mexyl (1-methylhexyl 5-chloro-8-quinolinoxy-acetic acid) stimulated the vacuolar transport of the GS-metolachlor conjugate and the glucoside conjugate of primisulfuron {2-[[[[[4,6-bis(difluoromethoxy)-2-pyrimidinyl]amino]carbonyl]amino]sulfonyl]benzoic acid}.

Concluding Remarks

Current and future studies on the purification, characterization, cloning, gene expression, and regulation of plant glutathione transferases will continue to advance our understanding of the properties and functions of these important enzymes. The ongoing elucidation of the crystal structures of plant GSTs involved in herbicide detoxification will enhance our ability to manipulate the resistance of crop plants to certain classes of herbicides and may facilitate the rational design of future herbicidal molecules. The discovery of additional endogenous substrates for specific plant GSTs is critical for a better understanding of the multiple functions of GSTs in higher plants. A better understanding of the mechanisms involved in stress recognition, signal transduction, and activation of defense genes will clarify our views on how safeners, pathogens, oxidative stress, heavy metals, and other stress factors act and/or interact at the molecular level to induce the genes of plant defense enzymes such as GSTs.

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch012

Acknowledgments

Research from the laboratory of the author was supported by research grants from the NRI/CGP/USDA (No. 9300565, 9601867 and 9800727) and Novartis Crop Protection Corporation (Formerly CIBA Plant Protection), Greensboro, NC. The author is especially thankful to Dr. Jingrui Wu for his collaboration and valuable contributions in the GST-related research projects conducted in the author's lab.

Literature Cited

1. Rennenberg, H. *Phytochemistry* **1982**, *21*, 2771-2781.
2. Meister, A. *Methods Enzymol.* **1995**, *252*, 26-30.
3. Hausladen A; Alscher, R.G. In *Antioxidants in Higher Plants*; Alscher, R.G. Hess, J.L., Eds.; CRC Press, Boca Raton, FL,1993; pp. 1-30.
4. Rennenberg, H.; Brunold, C. *Progress in Botany*, **1994**, *55*, 142-156.
5. Noctor, G.; Jouanin, L.; Foyer, C.H. In *Regulation of Enzymatic Systems Detoxifying Xenobiotics in Plants*; Hatzios, K.K., Ed.; NATO ASI Series, Kluwer Academic Publishers, Dordrecht, 1997, pp. 109-124.
6. Steinkamp, R; Renennberg, H. *Z. Naturforsch.* **1985**, *40c*, 29-33.
7. Macnicol, P.K. *Plant Sci.* **1987**, *53*, 229-235.
8. Lamoureux, G.L.; Rusness, D.G. In *Glutathione: Chemical, Biochemical and Medical Aspects;* D. Dolphin; R. Poulson; Avramovic, O. Eds.; Wiley, New York, 1989, pp. 153-196.
9. Zajc, A.; Neufeind, T.; Prade, L.; Reinemer, P.; Huber, R.; Bieseler, B. *Pestic. Sci.* **1999**, *55*, 248-252.
10. Edwards, R.: Dixon, R.A. *Phytochemistry* **1991**, *30*, 79-84.
11. Rauser, W.E. *Annu. Rev. Biochem.* **1990**, *59*, 61-86.
12. Stephens, J.C. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1990**, *41*, 553-575.

13. Kömives, T.; Gullner, G.; Kiraly, Z. In *Regulation of Enzymatic Systems Detoxifying Xenobiotics in Plants*; Hatzios K.K. Ed.; NATO ASI Series, Kluwer Academic Publishers, Dordrecht, 1997, pp. 85-96.
14. Link, G.; Tiller, K.; Baginsky, S. In *Regulation of Enzymatic Systems Detoxifying Xenobiotics in Plants*; Hatzios, K.K., Ed.; NATO ASI Series, Kluwer Academic Publishers, Dordrecht. 1997, pp. 125-137.
15. Wingate, V.P.M.; Lawton, M.A.; Lamb, C.J. *Plant Physiol.* **1988**, *87*, 206-210.
16. De Kok, L.J.; Maas, F.M.; Godeke, A.B.; Kuiper, P.J.C. *Plant and Soil* **1986**, *91*, 349-352.
17. Armstrong, R.N. *Adv. Enzymol.* **1993**, *69*, 1-44.
18. Marrs, K.A. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1996**, *47*, 127-158.
19. Rushmore, T.H.; Pickett, C.B. *J. Biol. Chem.* **1993**, *268*, 11475-11478.
20. Tsukuba, S.; Sato, K. *CRC Crit. Rev. Biochem. Mol. Biol.* **1992**, *27*, 337-384.
21. Lamoureux, G.L.; Shimabukuro; R.H.; Frear, D.S. In *Herbicide Resistance in Weeds and Crops;* J.C. Caseley, G.W. Cussans and R.K. Atkin Eds.; Butterworth-Heinemann, Oxford, 1991, pp. 227-261.
22. Lamoureux, G.; Rusness, D.G. In *Sulfur Nutrition and Assimilation in Higher Plants*; L.J. DeKok, I. Stulen, H. Rennenberg, C. Brunold and W. Rauser Eds.; SPB Academic Publishing, The Hague, 1993, pp. 221-237.
23. Timmerman, K.P. *Physiol. Plant.* **1989**, *77*, 323-342.
24. Cole, D.J.; Cummins, I.; Hatton, P.J.; Dixon, D.; Edwards, R. In *Regulation of Enzymatic Systems Detoxifying Xenobiotics in Plants;* Hatzios, K.K. Ed.; NATO ASI Series, Kluwer Academic Publishers, Dodrecht, 1997, pp. 139-154.
25. Nicolaus, B.; Sato, Y.; Wakabayashi, K.; Böger, P. In *Regulation of Enzymatic Systems Detoxifying Xenobiotics in Plants*; Hatzios, K.K. Ed.; NATO ASI Series, Kluwer Academic Publishers, Dodrecht, 1997, pp. 183-196.
26. Sommer, A.; Böger, P. *Pestic. Biochem. Physiol.* **1999**, *63*, 127-138.
27. Mars, K.A.; Alfenito, M.R.; Lloyd, A.M.; Walbot, V. *Nature* **1995**, *375*, 397-400.
28. McGonigle, B.; Lau, S-M., C.; O'Keefe, D.P. In *Regulation of Enzymatic Systems Detoxifying Xenobiotics in Plants*; Hatzios, K.K. Ed.; NATO ASI Series, Kluwer Academic Publishers, Dodrecht, 1997, pp. 9-18.
29. Sharma, Y.K.; Davis, K.R. *Plant Physiol.* **1994**, *105*, 1089-1096.
30. Hagen, G.; Uhrhammer, N.; Guilfoyle, T.J. *J. Biol. Chem.* **1988**, *263*, 7130-7139.
31. Kusaba, M.; Takahashi, Y.; Nagata, T. *Plant Physiol.* **1996**, *111*, 1161-1167.
32. Dudler, R.; Hertig, C.; Rebmann, G.; Bull, J.; Mauch, F. *Mol. Plant Microbe Interact.* **1991**, *4*, 14-18.
33. Taylor, J.L.; Fritzemeier, K.-H.; Häuser, I.; Kombrink, E.; Rohwer, F.; Schröder, M.; Strittmatter, G.; Hahlbrook, K. *Mol. Plant Microbe Interact.* **1990**, *3*, 72-77.
34. Bartling,D.; Radzio, R.; Steiner, U.; Weiler, E.W. *Eur. J. Biochem.* **1993**, *216*, 579-586.
35. Edwards, R. *Physiol. Plant.* **1996**, *98*, 594-604.
36. Bilang, J.; Sturm, A. *Plant Physiol.* **1995**, *109*, 253-260.
37. Jones, A.M. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1994**, *45*, 393-420.

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch012

38. Diesperger, H.; Sandermann, H. *Planta* **1979**, *146*, 643-648.
39. Cottingham, C.K.; Hatzios, K.K.; Meredith, S.A. *Z. Naturforsc*h. **1998**, *53c*, 973-979.
40. Dirr, H.W.; Reinemer, P.; Huber, R. *Eur. J. Bioche*m. **1994**, *220*, 645-661.
41. Wilce, M.C.; Board, P.G.; Fell, S.C.; Parker, M.W. *EMBO J.* **1995**, *14*, 2133-2143.
42. Droog, F.J.N.; Hooyakas, P.J.J.; van der Zaal, B.J. *Plant Physiol.* **1995**, *107*, 1139-1146.
43. Itzhaki, H.; Woodson, W.R. *Plant Mol. Biol.* **1993**, *22*, 43-58.
44. Droog, F. *J. Plant Growth Regul.* **1997**, *16*, 95-107.
45. Dixon, D.P.; Cummins,.J.; Cole, D.J.; Edwards, R. *Current Opinion in Plant Biol.* **1998**, *1*, 258-270.
46. Dean, J.V.; Devarenne, T.P.; Lee, I-S.; Orlofsky, L.E. *Plant Physiol.* **1995**, *108*, 985-994.
47. Dean, J.V.; Deverenne, T.P. *Physiol. Plant.* **1997**, *99*, 271-278.
48. Frear, D.S.; Swanson, H.R. *Phytochemistry* **1970**, *9*, 2123-2132.
49. Lay, M.M.; Casida, J.E. *Pestic. Biochem. Physiol.* **1976**, *6*, 442-456.
50. Dixon, D.A.; Cole, D.J.; Edwards, R. *Plant Mol. Biol.* **1998**, *36*, 75-87.
51. Mozer, T.J., Tiemeier, D.C.; Jaworksi, E.G. *Biochemistry* **1983**, *22*, 1068-1072.
52. Shah, D.M.; Hironake, C.M..; Wiegand, R.C.; Harding, E.I.; Krivi, G.G.; Tiemeier, C. *Plant Mol. Biol.* **1986**, *6*, 203-211.
53. Jepson, I; Lay, V.J.; Holt, D.C.; Bright, S.W.J.; Greenland, A.J. *Plant Mol. Biol.* **1994**, *26*, 1855-1866.
54. Rossini, L.; Jepson, I.; Greenland, A.J.; Sari-Gorla, M.; *Plant Physiol.* **1996**, *112*, 1595-1600.
55. Rossini, L., Pe, M.E., Frova, C.; Hein, K.; Sari-Gorla, M. *Mol. Gen Genet.* **1995**, *248*, 535-539.
56. Nicolaus, B.; Sato, Y.; Wakabayashi, K.; Böger, P. *Z. Naturforsch.* **1996**, *51c*, 342-354.
57. Holt, D.C.; Lay, V.J.; Clarke, E.D.; Dinsmore, A.; Jepson, I.; Bright, S.W.J.; Greenland, A.J. *Planta* **1995**, *196*, 295-302.
58. Grove, G.; Zarlango, R.P.; Timmerman, K.P.; Li, N-Q.;,Tam, M.F.; Tu, C-P.D. *Nucleic Acids Res.* **1988**, *16*, 425-438.
59. Moore, R.E.; Davies, M.S., O'Connell K.M.; Harding, E.I.; Wiegand, R.C.; Tiemeier, D.C. *Nucleic Acids Res.* **1986**, *14*, 7227-7235.
60. O'Connell, K.M.; Breaux, E.J.; Fraley, R.T.; *Plant Physiol.* **1988**, *86*, 359-363.
61. Marrs, K.A.; Walbot, V. *Plant Physiol.* **1997**, *113*, 93-100.
62. Irzyk, G.P.; Fuerst, E.P. *Plant Physiol.* **1993**, *102*, 803-810.
63. Irzyk, G.P.l Potter, S.; Ward, E.; Fuerst, E.P. *Plant Physiol.* **1995**, *107*, 311-312.
64. Dixon, D.P.; Cole, D.J.; Edwards, R. *Pestic. Sci.* **1997**, *50*, 72-82.
65. Cummins, I.; Cole, D.J.; Edwards, R. *Pestic. Biochem. Physiol.* **1997**, *59*, 35-49.
66. Pascal, S.; Debrauwer, L.; Ferte, M-P.; Anglade, P.; Rouimi, P.; Scalla, R. *Plant Sci.* **1998**, *134*, 217-226.
67. Pascal, S.; Scalla, R. *Physiol. Plant.* **1999**, *106*, 17-27.

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch012

68. Mauch, F.; Hertig, C.; Rebmann, G.; Bull, J.; Dudler, R. *Plant Mol. Biol.* **1991**, *16*, 1089-1091.
69. Mauch, F.; Dubler, R. *Plant Physiol.* **1993**, *102*, 1193-1201.
70. Gronwald, J.W.; Plaisance, K.L. *Plant Physiol.* **1998**, *117*, 877-892.
71. Wu, J.; Omokawa, H.; Hatzios, K.K. *Pestic. Biochem. Physiol.* **1996**, *54*, 220-229.
72. Wu, J.; Cramer, C.L.; Hatzios, K.K. *Physiol. Plant.* **1999**, *105*, 102-108.
73. Wu, J.; Cramer, C.L.; Hatzios, K.K. PGR 98-119 *Plant Physiol.* **1998**, *117*, 1126.
74. Wu, J.; Cramer, C.L.; Hatzios, K.K. PGR 98-120 *Plant Physiol.* **1998**, *117*, 1126.
75. Wu, J.; Cramer, C.L.; Hatzios, K.K. PGR 98-136 *Plant Physiol.* **1998**, *118*, 329.
76. Binh, L.T.; Oono, K. *Plant Physiol.* **1992**, *99*, 1146-1150.
77. Flury, T.; Adam, D.; Kreuz, K. *Physiol. Plant.* **1995**, *94*, 312-318.
78. Czarneca, E.; Nagano, R.T.; Key, J.L. *Mol. Cell Biol.* **1988**, *8*, 1113-1122.
79. Hagen, G.; Kleinschmidt, A.J.; Guilfoyle, T.J. *Planta*, **1984**, *162*, 147-153.
80. Ulmasoz, T.; Omiya, A.; Hagen, G.; Guilfoyle, T. *Plant Physiol.* **1995**, *108*, 919-927.
81. Hatton, P.J.; Edwards, R.; Cole, D.J.; Price, L.J. *Proc. Brighton Crop. Prot. Conf. Weeds*, **1995**, 281-286.
82. Anderson, M.P.; Gronwald, J.W. *Plant Physiol.* **1991**, *96*, 104-110.
83. Gray, J.A.; Balke, N.E.; Stoltenberg, D.E. *Pestic. Biochem. Physiol.* **1996**, *55*, 157-167.
84. Plaisance, K.L.; Gronwald, J.W. *Pestic. Biochem. Physiol.* **1999**, *63*, 34-49.
85. Frova, C., Sari-Gorla, M.; Pe, M.E.; Greenland, A.; Jepson, I.; Rossini, L. In *Regulation of Enzymatic Systems Detoxifying Xenobiotics in Plants*; Hatzios, K.K., Ed.; NATO ASI Series, Kluwer Academic Publishers, Dordrecht, 1997, pp. 171-181.
86. Jepson, I.; Holt, D.C.; Roussel, V.; Wright, S.Y.; Greenland, A.J. In *Regulation of Enzymatic Systems Detoxifying Xenobiotics in Plants*; Hatzios, K.K., Ed.; NATO ASI Series, Kluwer Academic Publishers, Dordrecht, 1997, pp. 313-323.
87. Schmitz, G.; Terres, K. *Mol. Gen. Genet.* **1992**, *233*, 269-277.
88. Wosnick, M.A.; Barnett, R.W.; Carlson, J.E. *Gene* **1989**, *76*, 153-160.
89. Chilton, M-D. *J. Agric. Food Chem.* **1988**, *36*, 3-5.
90. McGonigle, B; O'Keefe, D.P. PGR 98-079. *Plant Physiol.* **1998**, *117*, 332.
91. Andrews, C.J.; Jepson, I.; Skipsey, M.; Towson, J.K.; Edwards, R. PGR 97-050 *Plant Physiol.* **1987**, *113*, 1005.
92. Cole, D.J. *Pestic. Sci.* **1994**, *42*, 209-222.
93. Hatzios, K.K. In *Environmental Chemistry of Herbicides*; Grover, R; Fich, M. Eds.; CRC Press, Boca Raton, Florida, 1991, Vol. II, pp. 141-185.
94. *Regulation of Enzymatic System Detoxifying Xenobiotics in Plants*, Hatzios, K.K.. Ed.; NATO ASI Series, Kluwer Academic Publishers, Dordrecht, The Netherlands, 1997.
95. Kreuz, K.; Tommasini, R.; Martinoia, E. *Plant Physiol.* **1996**, *111*, 349-353.
96. Breaux, E.J. *Weed Sci.* **1987**, *35*, 463-468.
97. Breaux, E.J.; Patanella, J.E.; Sanders, E.F. *J. Agric. Food Chem.* **1987**, *35*, 474-478.

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch012

98. Farago, S.; Kreuz, K.; Brunold, C. *Pestic. Biochem. Physiol* **1993**, *47*, 199-205.
99. Reinemer, P.; Prade, L.; Hof, P.; Neuefeind, T.; Huber, R.; Zetl, R.; Palme, K.J.S.; Koelln, I.; Bartunik, H.D.; Bieseler, B. *J. Mol. Biol.* **1996**, *255*, 289-309.
100. Neuefeind, T.; Huber, R.; Dasenbrock, H.; Prade L.; Bieseler, B. *J. Mol. Biol.* **1997**, *274*, 446-453.
101. Neuefeind, T.; Huber, R.P.R.; Knäblein, J.; Prade, L.; Mann, K.; Bieseler, B. *J. Mol. Biol.* **1997**, *274*, 577-587.
102. Bieseler, B.; Fedtke, C.; Neufeind, T.; Etzel, W.; Prade, L.; Renemer, P. *Pflanzenschutz-Nachrichten Bayer* **1997**, *50*, 117-140.
103. Hatzios, K.K. *Z. Naturfrosch.* **1991**, *46c*, 819-827.
104. Kreuz, K. *Proc. Brighton Crop Prot. Conf. Weeds*, **1993**, 1249-1258.
105. Farago, S.; Brunold, C.; Kreuz, K. *Physiol. Plant.* **1994**, *91*, 537-542.
106. Irzyk, E.P.; Fuerst, E.P. In *Regulation of Enzymatic Systems Detoxifying Xenobiotics in Plants,* Hatzios, K.K., Ed.; NATO ASI Series, Kluwer Academic Publishers, Dordrect, 1997, pp. 155-170.
107. Hershey, H.P.; Stoner, T.D. *Plant Mol. Biol.* **1991**, *17*, 679-690.
108. De Veylder, L.; van Montagu, M.; Inze, D. *Plant Cell Physiol.* **1997**, *38*, 568-577.
109. Lamoureux, G.L.; Rusness, D.G. In *Pesticide Options 2000*, Ragsdale, N.N.; Kearney, P.C.; Plimmer, J.R., Eds.; American Chemical Society, Washington, DC., 1995, pp. 350-367.
110. Boydston, R.A.; Slife, F.W. *Weed Sci.* **1986**, *34*, 850-858.
111. Lamoureux, G.L.; Rusness, D.G. *Pestic. Biochem. Physiol.* **1986**, *26*, 323-342.
112. Sheppard, B.R.; McCall, P.J.; Stafford, L.E.; Zorner, P.S. *Pestic. Sci.* **1987**, *19*, 331-332.
113. Ezra, G.; Dekker, J.H.; Stephenson, G.R. 1985, *Weed Sci.* **1985**, *33*, 287-290.
114. Lamoureux, G.L.; Rusness, D.G. *Pestic. Biochem. Physiol.* **1989**, *34*, 187-204.
115. Lamoureux, G.L.; Rusness, D.G. *Pestic. Biochem. Physiol.* **1987**, *27*, 318-329.
116. Zhang, B.; Singh, K.B. *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 2507-2511.
117. Jepson, I., Andrews, C.J.; Roussel, V.; Skipsey, M.; Towson, J.K. *Abstr. Weed Science Soc. Am.* **1999**, *39*, 178.
118. Low, P.S.; Merida, J.R. *Physiol. Plant.* **1996**, *96*, 533-542.
119. Thenaken, R.; Levine, R.; Brisson, L.F.; Dixon, R.A.; Lamb, C.J. *Proc. Natl. Acad. Sci. USA*, **1995**, *92*, 4158-4163.
120. Schröder, P. In *Regulation of Enzymatic Systems Detoxifying Xenobiotics in Plants,* Hatzios, K.K., Ed.; NATO ASI Series, Kluwer Academic Publishers, Dordrecht, 1997, pp. 233-244.
121. Sandermann, H.; Haas, M.; Messner, B.; Pflugmacher, S.; Schröder, P.; Wetzel, A. In *Regulation of Enzymatic Systems Detoxifying Xenobiotics in Plants*, Hatzios, K.K., ed.; NATO ASI Series, Kluwer Academic Publishers, Dordrecht, 1997, pp. 211-231.
122. Blake-Kalff, M.M.A.; Randall, R.A.; Coleman, J.O.D. In *Regulation of Enzymatic Systems Detoxifying Xenobiotics in Plants*, Hatzios, K.K., ed.;

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch012

NATO ASI Series, Kluwer Academic Publishers, Dordrecht, 1997, pp. 245-259.

123. Lamoureux, G.L.; Rusness, D.G. *Z. Naturforsch*. **1991**, *46c*, 882-886.

124. Ishikawa, T. *Trends Bioch. Sci.* **1992**, *17*, 463-468.

125. Martinoia, E.; Grill, E.; Tommasini, R.; Kreuz, K.; Amhrein, N. *Nature* **1993**, *364*, 247-249.

126. Li, Z-S.; Zhao, Y.; Rhea, P.A. *Plant Physiol.* **1995**, *107*, 1257-1268.

127. Li, Z-S.; Zhen, R-G.; Rea, P.A. *Plant Physiol.* **1995**, *109*, 177-185.

128. Gaillard, C.; Dufaud, A.; Tommasini; R., Kreuz, K.; Amhrein, N.; Martinoia, E. *FEBS Lett.* **1994**, *352*, 219-221.

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch012

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch013

Chapter 13

Bacterial Glutathione *S*-Transferases and the Detoxification of Xenobiotics: Dehalogenation through Glutathione Conjugation and Beyond

Stéphane Vuilleumier

Institut für Mikrobiologie, ETH Zürich, CH–8092 Zürich, Switzerland

Several dozen sequences of bacterial glutathione S-transferases (GSTs) are now known, mainly as the result of whole genome sequencing efforts. So far, only a minority of bacterial GSTs have been characterized at the biochemical level. Some GSTs were shown to catalyze dehalogenation reactions, and several others also appear to be associated with metabolic pathways for the degradation of halogenated xenobiotic compounds. The modest sequence similarity observed between bacterial members of this protein family further suggests that they may catalyze a wide range of glutathione-dependent reactions within the conserved canonical GST structural framework. Characterization of the physiological function of bacterial GSTs and of the bacterial metabolism associated with glutathione conjugation reactions will be the key for the implementation of strategies for the detoxification of xenobiotics based on GSTs in bacteria.

Glutathione S-transferases (GSTs) are ubiquitous and versatile catalysts that detoxify xenobiotics and reactive endogenous compounds by conjugation to glutathione. GSTs from mammals have been extensively studied because of their association with cancer (*1*). In plants, the detoxification of pesticides, herbicides and environmental pollutants often involves GST enzymes (*2*). The resulting conjugates are then, either transported to the vacuole for storage and subsequent export (*3*), or processed further (*4*). Complete degradation (mineralization) of these conjugates appears to be rare. The occurrence of GST-dependent pathways in bacteria for the detoxification and degradation of xenobiotics, and of herbicides in particular, is documented in several

In Pesticide Biotransformation in Plants and Microorganisms; Hall, J., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2000.

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch013

reports. Several soil bacteria were found to contain GST activity and to catalyze the formation of glutathione conjugates of the herbicide alachlor (*5,6*), but the enzymes responsible for these transformations remain to be characterized. The importance of GSTs in the degradation of xenobiotics in the environment is further indicated by the detection of metabolites of herbicides in soil and groundwater that contain sulfur, such as the corresponding thiols, thioethers, sulfoxides, sulfones and sulfonic acids (*7,8*). However, the portion of this metabolism due to bacterial action remains to be determined. Even more remains to be learned about the regulation of GST expression, the uptake of pesticides and other hydrophobic GST substrates, and on the excretion, processing and toxicity of glutathione conjugates in bacteria. This is somewhat of a paradox considering the steadily increasing number of bacterial genes annotated as GSTs in sequence databases (Table I). This lack of knowledge about the physiological roles of bacterial GSTs also contrasts with the widespread interest in biodegradation and biodetoxification strategies relying on bacterial systems. Compared to plants, bacterial systems offer major technical advantages, including rapid growth, ease of molecular genetic methods, transformation by plasmids, and availability of comprehensive sequence data generated in genome sequencing projects. With regard to biodegradation approaches for remediation, bacteria often have the additional asset of permitting mineralization of the compounds of interest. This paper summarizes results and information on bacterial GSTs obtained since this subject was last reviewed (*9*). Aspects of the sequence and structure of bacterial GSTs and of the reactions catalyzed by their best characterized representatives will be addressed, as well as the physiological role of these enzymes, the cellular processes associated with GST catalysis, and the potential of bacteria expressing GSTs for remediation applications.

Sequence and structure similarity in bacterial GST enzymes

Enzymes with glutathione transferase activity are found in all realms of aerobic life. They appear to have evolved through both convergent and divergent pathways, as evidenced by differences in gene sequence, enzyme structure, and in amino acid residues involved in catalysis (*10*). Compared to representatives of the mammalian *alpha*, *mu* and *pi* classes of GSTs, bacterial and plant GSTs show extensive sequence variation and build only loosely defined groups (*11*). Nevertheless, similarity searches of newly sequenced putative bacterial GST genes almost exclusively retrieve hits to other GST sequences. Consequently, these new sequences are usually annotated as GSTs, despite the fact that the reactions catalyzed by the corresponding proteins are unknown. In addition, it seems unlikely that the glutathione-dependent stress proteins similar in sequence to the *N*-terminal domain of GSTs (*12*) actually catalyze detoxification reactions *strictu sensu*. The GST-like domain of one such protein, Ure2 from yeast, which displays prion-like behaviour, was recently proposed to stabilize the shorter prion-determining domain of Ure2 against prion formation (*13*). Clearly, more stringent criteria would be needed to discriminate between such protein sequences and those of GSTs involved in the detoxification of toxic

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch013

Table I. Sequence Similarity of Bacterial GSTs to Genomic Sets of GST Genes[a]

Name	Acc. No.[b]	Organism	Function or degradative gene cluster	Best hit (E value / % id.) [number of hits][c]	
				Escherichia coli	*P. aeruginosa*
DcmA	P21161	*Methylobacterium* sp. DM4	dichloromethane dehalogenase	YliJ (10^{-6} / 21%) [2]	GstA (10^{-9} / 21%) [4]
DcmA	P43387	*Methylophilus* sp. DM11	dichloromethane dehalogenase	-	GstN (10^{-8} / 24%) [4]
PcpC	Q03520	*Sphingomonas chlorophenolica*	tetrachlorohydroquinone reductase	-	GstE (10^{-5} / 29%) [3]
LigF	P30347	*Sphingomonas paucimobilis*	β-etherase	GT (10^{-9} / 21%) [2]	GstN (10^{-10} / 29%) [2]
Orf3	Q45073	*Burkholderia cepacia* AC1100	2,4,5-trichlorophenoxyacetic acid?	YfcG (10^{-52} / 21%) [4]	GstF (10^{-48} / 47%) [7]
BphK	Q59721	*Burkholderia* sp. LB400	chlorinated biphenyls?	GT (10^{-51} / 48%) [5]	GstB (10^{-13} / 27%) [8]
XylK	Q46153	*Cycloclasticus oligotrophus*	polycyclic aromatics?	GT (10^{-44} / 42%) [5]	GstQ (10^{-12} / 26%) [10]
OrfE3	O07878	*Sphingomonas* sp. RW1	chlorinated dibenzofurans and dioxins?	SspA (10^{-6} / 19%) [2]	GstQ (10^{-50} / 47%) [4]
NagL	O86043	*Pseudomonas* sp. U2	chlorinated gentisates?	SspA (10^{-10} / 23%) [1]	GstQ (10^{-48} / 50%) [6]
Orf3	O86923	*Sphingomonas* sp. RW5	chlorinated gentisates?	YliJ (10^{-12} / 24%) [4]	GstB (10^{-16} / 33%) [9]
MaiA	*AF109131*	*Sinorhizobium meliloti*	maleylacetoacetate isomerase?	GT (10^{-6} / 17%) [2]	GstP (10^{-39} / 41%) [8]
	D17523	plasmid pUOH109	2-haloacid dehalogenation?	YfcG (10^{-6} / 36%) [4]	GstF (10^{-40} / 37%) [6]
IsoI	*AJ249207*	*Rhodococcus* sp. AD45	epoxide ring-opening	-	GstK (10^{-26} / 31%) [2]
GstA	Q52828	*Rhizobium leguminosarum*	plant-bacterial interactions?	YfcG (10^{-13} / 26%) [6]	GstM (10^{-41} / 42%) [10]
GstB	P15214	*Proteus mirabilis*	antibiotic inactivation?	GT (10^{-65} / 54%) [5]	GstE (10^{-13} / 26%) [7]

[a] The *E. coli* genome contains 8 GST-like genes (*14*). The 17 GST-like genes detected in the preliminary version of the complete genome of *Pseudomonas aeruginosa* (*15*) were arbitrarily labelled GstA through GstQ in the order they appear in the 6.2 MB genome sequence. [b] Accession numbers are from the Swissprot/Trembl protein database except those in italics. [c] Significant hits with an E-value of < 10^{-3} in a GAPPED-BLAST search (*16*) and which extend over 100 amino acids are shown. Identity in percent is calculated for the shorter of the two sequences compared.

chemicals. Nevertheless, the flood of new GST-like sequences resulting from genome DNA sequencing projects in prokaryotes carries two important messages. First, these studies indicate that a single bacterium may contain a large set of GST genes whose size and content may vary significantly (Table I). Second, many new bacterial GST-like genes, listed in Table I, appear to be associated with operons and gene clusters involved in the degradation (and often the dehalogenation) of xenobiotics.

The 4.6 megabase genome of *Escherichia coli* (*17*) contains 8 GST-like genes (*14*), compared to 17 for the *Pseudomonas aeruginosa* genome comprised of 6.2 megabases (*15*). Interestingly, only 4 of the detected GST genes appear to have close homology (>40% sequence identity at the protein level) in both genomes. In addition, GST sequences found in biodegradation-associated gene clusters from bacteria are often similar to only a subset of the GST sequences encoded in a given genome (Table I). Thus, overall sequence variability within the bacterial GST family of proteins may well be much larger than that found in a single genome. Nevertheless, the *Pseudomonas* set appears to be more closely related overall to bacterial biodegradation-associated GSTs than the *E. coli* one (Table I).

In no case has the physiological role of a GST detected by whole-genome sequencing yet been determined. This may continue to hold true in the near future for several reasons. First, the observed low level of sequence identity in bacterial GSTs makes it unrealistic to decide on the function of a given GST from sequence alone. Second, bacterial GSTs of known function catalyze varied reactions (Table I) and often lack activity with standard model GST substrates such as 1-chloro-2,4-dinitrobenzene (CDNB). Third, a phenotype may be difficult to determine in specific GST knockout mutants, since the large sets of GST-like genes found in genomes may lead to partial complementation of the function of the disrupted gene. In addition, a classical growth phenotype in a GST-minus mutant is not expected *a priori*, because mutants of *E. coli* lacking glutathione are still viable. Insights into the role of the proteins encoded by putative GST genes detected in genome sequencing projects will most likely arise from gene expression studies under a wide variety of conditions.

It is important to note that the extensive sequence variation in the GST family does not appear to significantly affect the structural framework within which GSTs catalyze glutathione conjugation reactions (Figure 1). For example, a GST from *A. thaliana* involved in herbicide tolerance (*19*), two bacterial enzymes of known structure from *E. coli* (*22*) and *Proteus mirabilis* (*18*), and the human GST *theta* 2-2 enzyme (*20*) used as a model for the dichloromethane-active human GST *theta* 1-1 enzyme (hGSTT1-1) (*21*), can be structurally aligned over 117 residues. The structural similarity between these proteins appears even closer in pairwise comparisons, despite sequence identity below 20% and only 15 identical residues in the four proteins (Table II). Thus, the GST structural framework has served as an evolutionarily stable scaffold for the development of detoxification catalysts relying

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch013

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch013

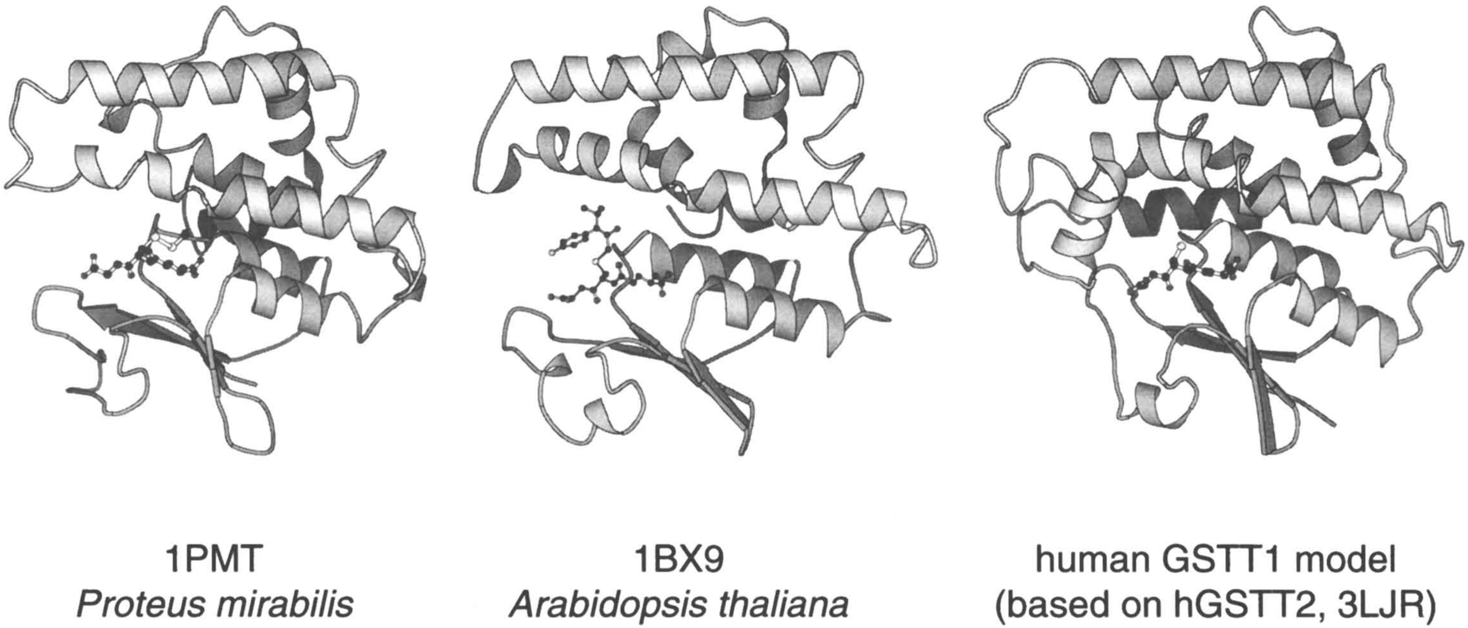

1PMT
Proteus mirabilis
1BX9
Arabidopsis thaliana
human GSTT1 model
(based on hGSTT2, 3LJR)

Figure 1. Comparison of the aligned structures of the bacterial GST from P. mirabilis ((18); enzyme-GSH mixed disulfide), the alachlor-active GST from Arabidopsis thaliana ((19); FOE-4059 glutathione conjugate) and a model of the human GSTT1 (20) based on the human GSTT2 structure (21).

on glutathione conjugation. This fact is already being exploited in the laboratory, with the application of methods of phage display (*23,24*) and DNA shuffling (*25*) for the selection of GST catalysts with the desired specificity in bacteria.

Table II. Percent Structural vs. Sequence Similarity Between Glutathione S-Transferases of Known X-Ray Structure

Organism (PDB entry)	*E. c.*	*P. m.*	*A. th.*	*hGSTT1*
*Escherichia coli (*1AOF)	[a]	54	18	20
Proteus mirabilis (1PMT)	98		20	16
Arabidopsis thaliana (1BX9)	77	70		18
human GSTT1 model (3LJR[b])	70	71	74	

[a] Left of diagonal: aligned residues (%); right of diagonal: identical residues (%).
[b] hGSTT1 model based on the the 55% identical hGSTT2 X-ray structure (*21*).

Bacterial GSTs of Known Function

Bacterial dichloromethane dehalogenases are the oldest known examples of bacterial GSTs (*26*). They are perhaps also the most atypical, both by sequence and by the reactions they catalyze (Figure 2A). Protein engineering studies of the enzyme from *Methylophilus* sp. DM11 (*27-29*) have enabled (to some degree) the elucidation of its catalytic determinants. Its reaction mechanism is still poorly understood, but thought to involve a reactive, short-lived intermediate, S-chloromethylglutathione. Recent work has addressed the toxicity associated with GST-dependent turnover of dichloromethane (DCM) observed for the closest homologs of bacterial DCM dehalogenases, the GSTT1-1 enzymes from mammals (*30*). This toxicity was surprising since no detrimental effects were observed in methylotrophic bacteria growing with DCM as the unique carbon source by virtue of their DCM dehalogenase. It was recently shown that the rat GSTT1-1 enzyme has much lower affinity for DCM than bacterial DCM dehalogenases, despite affording a higher turnover rate at very high substrate concentrations (*31*). Furthermore, expression of rat GSTT1-1 in the presence of DCM (unlike that of bacterial DCM dehalogenase) was toxic in the *Methylobacterium* sp. DM4 background, and mutagenic in a *Salmonella* Ames tester strain (*31*). Clearly, only bacterial versions of DCM-active GSTs allow the safe degradation of this toxic compound. The comparison of DCM-converting GST enzymes from mammals and bacteria thus provides a vivid example that proteins with similar sequences that catalyze the same transformation, may nonetheless have strikingly different consequences for the host organism.

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch013

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch013

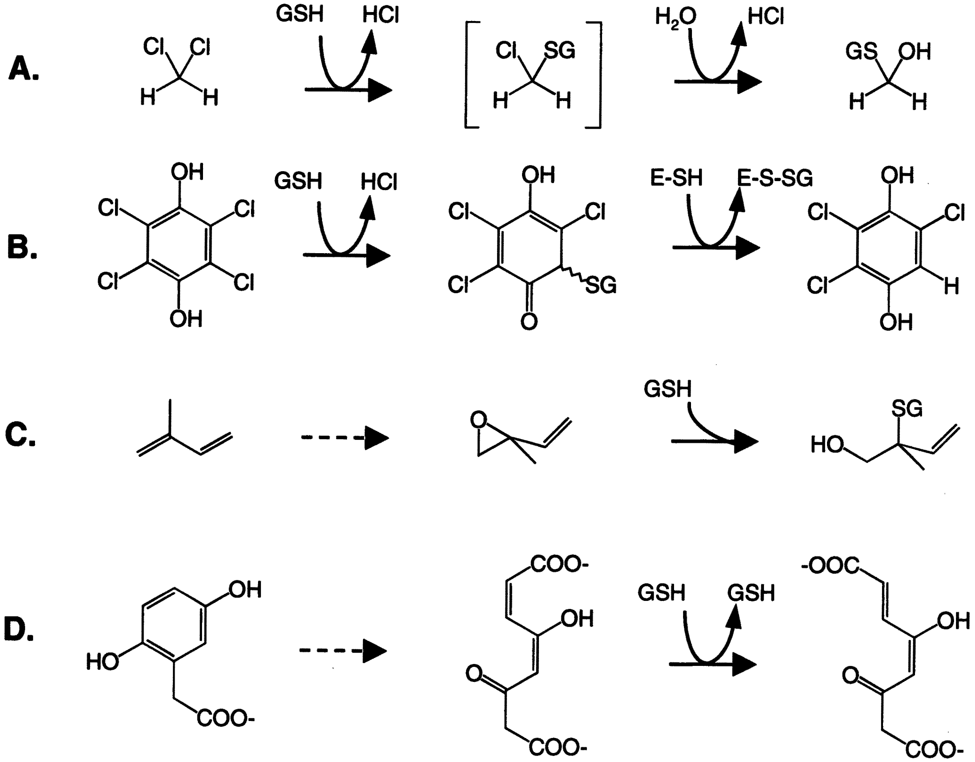


Figure 2. Some reactions catalysed by different bacterial GSTs. A) dichloromethane dehalogenation, B) reductive dehalogenation of chlorinated hydroquinones, C) epoxide ring-opening, D) carbon-carbon double bond isomerization.

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch013

Another atypical GST from bacteria is the reductive dehalogenase tetrachlorohydroquinone reductase (PcpC) from *Sphingomonas chlorophenolica*, which catalyzes the stepwise glutathione-dependent dehalogenation of tetrachlorohydroquinone to dichlorohydroquinone with concomitant oxidation of glutathione (Figure 2B). The PcpC sequence is one of the most divergent within known bacterial GSTs (*32*), together with that of the β-etherase LigF from *Sphingomonas paucimobilis* involved in lignin degradation (*33*) (Table I). The sequence of the functionally related 2,5-dichlorohydroquinone reductive dehalogenase (LinD) involved in γ-hexachlorocyclohexane degradation (*34*) is even more distantly related. These three GST-like proteins all oxidize glutathione during catalysis, thus demonstrating the existence of other functions of bacterial GSTs beyond conjugation. The PcpC enzyme was recently characterized in more detail (*32,35,36*). An unexpected finding was the transient formation of a mixed disulfide of glutathione with residue cysteine 13 of the protein during turnover (*35*). The thiol form of the enzyme is then regenerated by glutathione-dependent reduction, yielding glutathione disulfide. Interestingly, the structures of glutaredoxins, proteins which catalyze reversible oxidation/reduction of protein disulfide groups and glutathione-containing mixed disulfides (*37*), share a common structural fold with GSTs despite negligible sequence identity (*38*). A mixed disulfide between enzyme and glutathione was observed in the X-ray structure of the *Proteus mirabilis* glutathione S-transferase ((*18*), Figure 1), and the GST from *Rhizobium leguminosarum* may also form a mixed disulfide with glutathione (*39*). It is not yet known, however, whether a mixed disulfide with glutathione is relevant for the reaction catalyzed by GSTs other than PcpC.

An exciting recent development in the field of bacterial GSTs was the characterization of the biodegradative pathway for the degradation of isoprene and chlorinated ethenes, in which a GST enzyme features prominently (*40-42*). The key step in this pathway involves the GST-catalyzed opening of an epoxide ring (Figure 2C). Somewhat surprisingly, the bacterium effecting these transformations is a gram-positive *Rhodococcus* strain. With a few exceptions, gram-positive bacteria appear to be devoid of glutathione (*43*). The enzyme responsible for the epoxide ring opening reaction was purified and characterized, and the corresponding *isoI* gene cloned and sequenced ((*41*), see Table I). Sequence similarity of the IsoI GST to other proteins was modest and limited to only a few other GST sequences. Another glutathione-dependent epoxide ring opening enzyme, FosA, affords fosfomycin resistance in the host bacteria (*44,45*). In contrast to the *Rhodococcus* enzyme, however, this enzyme belongs to a completely different structural class of protein catalysts, i. e., metalloenzymes that include glyoxalase and extradiol dioxygenases, with no detectable sequence similarity to *bona fide* GSTs. The *Rhodococcus* enzyme displays a K_m for glutathione well above 10 mM, in contrast to a high affinity for isoprene monoxide and 1,2-*cis*-dichloroepoxyethane (both K_m = 0.1 mM). The latter substrate is postulated to decay spontaneously to oxaldehyde by HCl elimination from the glutathione adduct, followed by hydrolysis and subsequent elimination of a second molecule of HCl (*40*). Thus, this enzyme is both a *bona fide* glutathione conjugating

GST and a dehalogenase. The route for the further degradation of the stable glutathione conjugate of isoprene epoxide in *Rhodococcus* is still unknown, although some of the genes found at the same locus as the GST gene may be involved in this process (*41*). Further investigations with this system should therefore yield novel insights on the catabolism of glutathione conjugates in bacteria.

The dependence of enzyme-catalyzed carbon-carbon double bond isomerization reactions on glutathione was already investigated in detail in the sixties and seventies (*46-48*). An unexpected connection of glutathione-dependent isomerases with GST-catalyzed dehalogenation reactions recently became apparent. The human GST gene defining a new class of GSTs, *zeta*, was demonstrated to encode an enzyme catalyzing the dehalogenation of 2-haloacids (*49,50*). Surprisingly, the same gene was also shown to complement a gene defect in the metabolism of tyrosine at the level of homogentisate oxidation (*51*). The corresponding enzyme had the function of a maleylacetoacetate isomerase ((*52*), Figure 2D). Genes encoding proteins similar to this bifunctional human GST were found in bacterial operons involved in the degradation of aromatic compounds such as homogentisate and gentisate (*53-56*), Table I). Since the corresponding bacteria were investigated for their ability to degrade halogenated aromatics, this suggests the possibility that the corresponding proteins, as the human enzyme, may act both as dehalogenases and as isomerases. The verification of this hypothesis, unfortunately, requires relatively unstable substrates that are not commercially available. However, the repeated findings of GST-like genes in gene clusters involved in the ring opening of aromatic compounds by dioxygenases certainly indicate that the corresponding gene products play a role in the catabolism of aromatic compounds.

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch013

GST-associated metabolism in bacteria

With respect to GST-dependent bacterial transformation of xenobiotics, many important questions remain to be investigated. Can glutathione conjugates be excreted, or indeed taken up by bacteria, or are they mainly metabolized intracellularly? What are the enzymes and proteins required in these processes? What is the toxicity of xenobiotics and of their corresponding glutathione conjugates and degradation products to bacteria? Do such compounds elicit the induction of other genes?

These questions can be addressed experimentally, as several recent studies have begun to demonstrate. The penetration of xenobiotics into bacterial cells and the formation and localization of glutathione conjugates can be traced using labelled substrates (*6,57*). The occurrence and role of glutathione conjugate transporters can be investigated using uncouplers and inhibitors (*58*). In contrast to the situation in plants, however, only a few studies of the catabolism of glutathione conjugates in bacteria have yet been reported. The glutathione conjugate of alachlor was proposed to be processed by a specific protease, γ-glutamyltranspeptidase, and by cysteine β-lyase, since these activities were detected in cell-free extracts of alachlor-conjugating bacteria (*6*).

Another overlooked aspect of the metabolism associated with GST-dependent degradation of xenobiotics regards the toxicity of GST substrates and of their corresponding glutathione conjugates and metabolites. Glutathione conjugation is generally assumed to cause the detoxification of electrophilic toxic chemicals, but some glutathione conjugates, such as that of CDNB, are actually more toxic than the parent compounds (*59*). In the case of GST-mediated DCM catabolism, the postulated short-lived S-chloromethylglutathione conjugate is a known DNA alkylating agent thought to be responsible for the mutagenicity associated with DCM (*60*). Mutants of *Methylobacterium* sp. DM4, recently obtained by minitransposon mutagenesis, lost the ability to grow on DCM as the sole carbon source, but still possessed an active DCM dehalogenase/GST. The genes carrying an insertional lesion in these mutants were involved in DNA repair (unpublished results).

Glutathione-conjugated products of GST-catalyzed reactions may also have a signalling function in cellular metabolism. For example, the Kef proteins of *E. coli,* induced by certain glutathione conjugates, cause potassium efflux with associated proton influx, leading to protection against the toxicity of electrophilic compounds (*61*).

Finally, the uptake of GST substrates by bacteria is another topic that deserves closer attention in the future. Preferential uptake of a GST substrate by some bacteria may also strongly determine GST-based metabolism, as observed by the growth behavior of different DCM-degrading methylotrophic bacteria. When DCM was present in limiting amounts as the sole carbon source, the measured growth rate was significantly higher than that expected from the amount and specific activity of the DCM dehalogenase/GST in the case of *Methylobacterium* sp. DM4, but not with *Methylophilus* sp. DM11 (*62*). This tentatively suggests that a specific uptake system for DCM was induced in the *Methylobacterium* strain under these conditions. Interestingly, a high-affinity substrate uptake system was recently described in detail for a methylotrophic bacterium degrading short-chain alkylamides and urea (*63*).

Applications of bacteria expressing biodegradative GST enzymes

Considering the wide scope of reactions catalyzed by bacterial GSTs of known function, such proteins and the bacteria that express them appear as attractive candidates for biodegradation and biodetoxification applications. Bacteria with efficient glutathione-based degradation pathways also have potential in combination with phytoremediation approaches, which involve the use of plants to degrade persistent xenobiotics (*64*). Enhanced detoxification of xenobiotics by bacteria in soil in the presence of plants has been repeatedly observed in field studies. In addition, bacteria may be well-suited to further degrade potentially toxic glutathione conjugates of xenobiotics produced by plants. For remediation applications based on GST-expressing bacteria to be developed, however, an increased knowledge of the function and substrate specificity of bacterial GSTs is required. The screening of soil bacterial isolates to detect glutathione-dependent transformation of target xenobiotics (*6*) should constitute a choice approach towards this goal. Concomitantly and

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch013

importantly, the pathways of xenobiotic degradation involving GSTs, the metabolic processes associated with the toxicity of the electrophilic substrates and of the corresponding glutathione conjugates, as well as the distribution of bacteria with prominent GST-dependent catabolic pathways in different environments also need to be characterized. Clearly, future work in this area in the next few years will set the stage for the implementation of GST-based strategies for the detoxification of xenobiotics using bacterial systems.

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch013

Acknowledgements

Funding by grant 31-50602.97 of the Swiss National Research Foundation is gratefully acknowledged.

Literature Cited

1. McLellan, L. I.; Wolf, C. R. *Drug Res. Updates* **1999**, *2*, 153-164.
2. Marrs, K. A. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1996**, *47*, 127-158.
3. Rea, P. A.; Li, Z.-D.; Lu, Y.-P.; Drozdowicz, Y. M.; Martinoia, E. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1998**, *49*, 727-760.
4. Lamoureux, G. L.; Rusness, D. G. In *Sulfur Nutrition and Assimilation in Higher Plants*; De Kok, L. J.; Stulen, I.; Rennenberg, H.; Brunold, C.; Rauser, W. E., Eds.; SPB Academic Publishing: The Hague, 1993; pp 221-237.
5. Zablotowicz, R. M.; Hoagland, R. E.; Locke, M. A. In *Bioremediation Through Rhizosphere Technology*; Anderson, T. A.; Coats, J. R., Eds.; American Chemical Society: Washington, DC, 1994; Vol. 563, pp 184-198.
6. Zablotowicz, R. M.; Hoagland, R. E.; Locke, M. A.; Hickey, W. J. *Appl. Environ. Microbiol.* **1995**, *61*, 1054-1060.
7. Feng, P. C. C. *Pest. Biochem. Physiol.* **1991**, *40*, 136-142.
8. Field, J. A.; Thurman, E. M. *Environ. Sci. Technol.* **1996**, *30*, 1413-1418.
9. Vuilleumier, S. *J. Bacteriol.* **1997**, *179*, 1431-1441.
10. Armstrong, R. N. *Chem. Res. Toxicol.* **1997**, *10*, 2-18.
11. Snyder, M. J.; Maddison, D. R. *DNA Cell Biol.* **1998**, *16*, 1373-1384.
12. Koonin, E. V.; Mushegian, A. R.; Tatusov, R. L.; Altschul, S. F.; Bryant, S. H.; Bork, P.; Valencia, A. *Protein Sci.* **1994**, *3*, 2045-2054.
13. Perrett, S.; Freeman, S. J.; Butler, P. J. G.; Fersht, A. R. *J. Mol. Biol.* **1999**, *290*, 331-345.
14. Vuilleumier, S.; Gisi, D.; Stumpp, M. T.; Leisinger, T. *Clin. Chem. Enzymol. Commun.* **1999**, *8*, 367-378.
15. *Pseudomonas Genome Project*, URL http://pseudomonas.genome.com.
16. Altschul, S. F.; Madden, T. L.; Schaeffer, A. A.; Zhang, J.; Zhang, Z.; Miller, W.; Lipman, D. J. *Nucleic Acids Res.* **1997**, *25*, 3389-3402.
17. Blattner, F. R.; Plunkett, G., III; Bloch, C. A.; Perna, N. T.; Burland, V.; Riley, M.; Collado Vides, J.; Glasner, J. D.; Rode, C. K.; Mayhew, G. F.; Gregor, J.;

Davis, N. W.; Kirkpatrick, H. A.; Goeden, M. A.; Rose, D. J.; Mau, B.; Shao, Y. *Science* **1997**, *277*, 1453-1462.

18. Rossjohn, J.; Polekhina, G.; Feil, S. C.; Allocati, N.; Masulli, M.; Di Ilio, C.; Parker, M. W. *Structure* **1998**, *6*, 721-734.
19. Prade, L.; Huber, R.; Bieseler, B. *Structure* **1998**, *6*, 1445-1452.
20. Rossjohn, J.; McKinstry, W. J.; Oakley, A. J.; Verger, D.; Flanagan, J.; Chelvanayagam, G.; Tan, K.-L.; Board, P. G.; Parker, M. W. *Structure* **1998**, *6*, 309-322.
21. Flanagan, J. U.; Rossjohn, J.; Parker, M. W.; Board, P. G.; Chelvanayagam, G. *Proteins Struct. Funct. Genet.* **1998**, *33*, 444-454.
22. Nishida, M.; Harada, S.; Noguchi, S.; Satow, Y.; Inoue, H.; Takahashi, K. *J. Mol. Biol.* **1998**, *281*, 135-147.
23. Mannervik, B.; Cameron, A. D.; Fernandez, E.; Gustafsson, A.; Hansson, L. O.; Jemth, P.; Jiang, F. Y.; Jones, T. A.; Larsson, A. K.; Nilsson, L. O.; Olin, B.; Pettersson, P. L.; Ridderstrom, M.; Stenberg, G.; Widersten, M. *Chem. Biol. Int.* **1998**, *112*, 15-21.
24. Demartis, S.; Huber, A.; Viti, F.; Lozzi, L.; Giovannoni, L.; Neri, P.; Winter, G.; Neri, D. *J. Mol. Biol.* **1999**, *286*, 617-633.
25. Hansson, L. O.; Bolton Grob, R.; Massoud, T.; Mannervik, B. *J. Mol. Biol.* **1999**, *287*, 265-276.
26. Leisinger, T.; Bader, R.; Hermann, R.; Schmid-Appert, M.; Vuilleumier, S. *Biodegradation* **1994**, *5*, 237-248.
27. Vuilleumier, S.; Leisinger, T. *Eur. J. Biochem.* **1996**, *239*, 410-417.
28. Vuilleumier, S.; Sorribas, H.; Leisinger, T. *Biochem. Biophys. Res. Commun.* **1997**, *238*, 452-456.
29. Cai, B.; Vuilleumier, S.; Wackett, L. P. *Ann. NY Acad. Sci.* **1998**, *864*, 210-213.
30. Thier, R.; Taylor, J. B.; Pemble, S. E.; Humphreys, W. G.; Persmark, M.; Ketterer, B.; Guengerich, F. P. *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 8576-8580.
31. Gisi, D.; Leisinger, T.; Vuilleumier, S. *Arch. Toxicol.* **1999**, *73*, 71-79.
32. McCarthy, D. L.; Navarrete, S.; Willett, W. S.; Babbitt, P. C.; Copley, S. D. *Biochemistry* **1996**, *35*, 14634-14642.
33. Masai, E.; Katayama, Y.; Kubota, S.; Kawai, S.; Yamasaki, M.; Morohoshi, N. *FEBS Lett.* **1993**, *323*, 135-140.
34. Miyauchi, K.; Suh, S. K.; Nagata, Y.; Takagi, M. *J. Bacteriol.* **1998**, *180*, 1354-1359.
35. McCarthy, D. L.; Louie, D. F.; Copley, S. D. *J. Amer. Chem. Soc.* **1997**, *119*, 11337-11338.
36. Copley, S. D. *Curr. Op. Chem. Biol.* **1998**, *2*, 613-617.
37. Nordstrand, K.; Åslund, F.; Holmgren, A.; Otting, G.; Berndt, K. D. *J. Mol. Biol.* **1999**, *286*, 541-552.
38. Martin, J. L. *Structure* **1995**, *3*, 245-250.
39. Alkafaf, N. K. T.; Yeoman, K. H.; Wexler, M.; Hussain, H.; Johnston, A. W. B. *Microbiology* **1997**, *143*, 813-822.
40. van Hylckama Vlieg, J. E. T.; Kingma, J.; van den Wijngaard, A. J.; Janssen, D. B. *Appl. Environ. Microbiol.* **1998**, *64*, 2800-2805.

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch013

41. van Hylckama Vlieg, J. E. T. *Ph. D. Thesis, Rijksuniversiteit, Groningen*, **1999**.
42. van Hylckama Vlieg, J. E. T.; Kingma, J.; Kruizinga, W.; Janssen, D. B. *J. Bacteriol.* **1999**, *181*, 2094-2101.
43. Sherrill, C.; Fahey, R. C. *J. Bacteriol.* **1998**, *180*, 1454-1459.
44. Armstrong, R. N. *Curr. Op. Chem. Biol.* **1998**, *2*, 618-623.
45. Armstrong, R. N.; Bernat, B. A. In *Enzymatic Mechanisms*; Frey, P. A.; Northrop, B., Eds.; IOS Press: Amsterdam, 1999; pp 215-223.
46. Lack, L. *J. Biol. Chem.* **1961**, *236*, 2835-2840.
47. Crawford, R. L.; Frick, T. D. *Appl. Environ. Microbiol.* **1977**, *34*, 170-174.
48. Seltzer, S. In *Glutathione, Chemical, Biochemical and Medical Aspects*; Dolphin, D.; Avramovic, O.; Poulson, R., Eds.; Wiley-Interscience: New York, 1989; Vol. B, pp 733-751.
49. Tong, Z.; Board, P. G.; Anders, M. W. *Biochem. J.* **1998**, *371*, 371-374.
50. Tong, Z.; Board, P. G.; Anders, M. W. *Chem. Res. Toxicol.* **1998**, *11*, 1332-1338.
51. Fernández-Cañón, J. M.; Peñalva, M. A. *J. Biol. Chem.* **1998**, *273*, 329-337.
52. Fernández-Cañón, J. M.; Hejna, J.; Reifsteck, C.; Olson, S.; Grompe, M. *Genomics* **1999**, *58*, 263-269.
53. Armengaud, J.; Timmis, K. N. *Eur. J. Biochem.* **1997**, *247*, 833-842.
54. Fuenmayor, S. L.; Wild, M.; Boyes, A. L.; Williams, P. A. *J. Bacteriol.* **1998**, *180*, 2522-2530.
55. Werwath, J.; Arfmann, H.-A.; Pieper, D. H.; Timmis, K. H.; Wittich, R.-M. *J. Bacteriol.* **1998**, *180*, 4171-4176.
56. Milcamps, A.; deBruijn, F. J. *Microbiology* **1999**, *145*, 935-947.
57. Coleman, J. O. D.; Randall, R.; Blake-Kalff, M. M. A. *Plant Cell Environ.* **1997**, *20*, 449-460.
58. Kalużna, A.; Bartosz, G. *Biochem. Mol. Biol. Int.* **1997**, *43*, 161-171.
59. Lee, H. C.; Toung, Y. P. S.; Tu, Y. S. L.; Tu, C. P. D. *J. Biol. Chem.* **1995**, *270*, 99-109.
60. Hashmi, M.; Dechert, S.; Dekant, W.; Anders, M. W. *Chem. Res. Toxicol.* **1994**, *7*, 291-296.
61. Ferguson, G. P. *Trends Microbiol.* **1999**, *7*, 242-247.
62. Gisi, D.; Willi, L.; Traber, H.; Leisinger, T.; Vuilleumier, S. *Appl. Environ. Microbiol.* **1998**, *64*, 1194-1202.
63. Mills, J.; Wyborn, N. R.; Greenwood, J. A.; Williams, S. G.; Jones, C. W. *Eur. J. Biochem.* **1998**, *251*, 45-53.
64. Salt, D. E.; Smith, R. D.; Raskin, I. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1998**, *49*, 643-668.

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch013

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch014

Chapter 14

Enzymological Studies on Recombinant Isoforms of Glutathione *S*-Transferase from Corn

Alfred Sommer and Peter Böger

Department of Planet Physiology and Biochemistry, University of Konstanz, Konstanz, D–78457, Germany

Thiadiazolidines (5-arylimino-3,4-tetramethylene-1,3,4-thiadiazolidin-2-ones) are converted in plants into isomeric 4-aryl-1,2-tetramethylene-1,2,4-triazolidin-3-one-5-thiones (triazolidines). The latter are strong peroxidizing compounds, and the isomerization is catalyzed by glutathione S-transferase (GST). This prompted us to study in detail the properties of GST isoforms from corn *(Zea mays)* which were produced by *E. coli* transformed with expression vectors carrying a 6xHis-tag code to allow affinity purification of the enzymes. These isoforms are dimers with subunits of 29/29-, 27/29-, 26/26-, and 27/27-kDa for GST I, II, III and IV, respectively. Besides assays for GST-activities [1-chloro-2,4-dinitrobenzene (CDNB) and 2-chloro-*N*-(2,6-dimethylphenyl)-*N*-(1*H*-pyrazol-1-ylmethyl) acetamide (metazachlor) conjugation], additional modification of unsaturated aldehydes and glutathione peroxidase activity were also measured. The above-mentioned isomerization is catalyzed by GST II (27/29) and GST IV (27/27); also other enzymic activities toward the substrates tested were the highest with isoforms containing the 27-kDa subunit.

Glutathione S-transferases (GSTs , EC 2.5.1.18) are a group of enzymes that catalyze the conjugation of a wide range of hydrophobic, electrophilic, usually cytotoxic substrates with the tripeptide glutathione (γ-glutamyl-cysteinyl-glycine, GSH). Polar nontoxic peptide conjugates are then accessible for further metabolism (*1*). It becomes clear that conjugation activity is not their only role, and this paper will examine and discuss additional catalytic activities. Almost all organisms possess multiple GST isoenzymes which are classified in *alpha*, *mu*, *pi*, *sigma*, *tau* and *theta* classes (*1 - 3*). Plant GSTs were first identified and have been intensively studied because of their ability to detoxify herbicides (*1, 4, 5*). Plants with higher GST activity levels may withstand exposure to certain herbicides which kill susceptible species (*1, 6, 7*). Enzymatic activities towards different classes of herbicides such as chloroacetamides, e.g., {acetochlor [2-chloro-*N*-(ethoxymethyl)-*N*-(2-ethyl-6-methylphenyl)acetamide], alachlor [2-chloro-*N*-(2,6-diethylphenyl)-*N*-(methoxymethyl)acetamide] metazachlor [2-chloro-*N*-(2,6-dimethylphenyl)-*N*-(1*H*-pyrazol-1-ylmethyl) acetamide] or metolachlor [2-chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-(2-methoxy-1-methylethyl) acetamide]}, chloro-*S*-triazines (e.g., atrazine [6-chloro-*N*-ethyl-*N'*-(1-methylethyl)-1,3,5-triazine-2,4-diamine]), thiocarbamates, e.g., EPTC (S-ethyl dipropyl carbamothioate) sulfoxide, (*8*) and peroxidizing herbicides, have been investigated (*9*).

Peroxidizing herbicides such as oxyfluorfen [2-chloro-1-(3-ethoxy-4-nitrophenoxy)-4-(trifluoromethyl)benzene] or cyclic imides, lead to rapid phytotoxic degradation of plant cell constituents (*10*). In corn, isomerization of thiadiazolidine to peroxidizing triazolidine herbicides is catalyzed by GSTs, resulting in bioactivation of these herbicides (*11*).

Most cytosolic plant GSTs in crops have been classified (*3, 12*). Class type I (*theta*), type II (*zeta*), type III (*tau*) and type IV were derived from phylogenetic DNA analysis. Presently, eight cytosolic GST isoenzymes in corn have been described. Seven dimeric isoforms are involved in herbicide metabolism. These isoenzymes include five different subunits: GST29 (29 kDa), GST27 (27 kDa), GST26 (26 kDa; 3 partly different cDNA sequences were determined), all belonging to class *theta*, *Zm* GST V (class *tau*; 28.5 kDa) and *Zm* GST VI (class *tau*; 27.5 kDa). Some cDNA clones of corn GST subunits have been isolated (GST29 (*13, 14*), GST27 (*13, 15*), GST26 (*2, 16, 17*) and *Zm* GST V (*12*)). GST I is a constitutive (and somewhat safener-inducible) homodimer of two GST29 subunits and shows activity towards alachlor, atrazine and CDNB (*1, 11, 18*). This latter isoenzyme also catalyzes the isomerization of peroxidizing thiadiazolidin-one herbicides to the more active triazolidin-one-thiones (*11, 19, 20*). GST II is a safener-inducible {by flurazole [phenylmethyl-2-chloro-4-(trifluoromethyl)-5-thiazolecarboxylate] and dichlormid [2,2-dichloro-*N*-*N*-di-2-propenylacetamide] (*1*)} heterodimer (a GST29 and a GST27 subunit) which shows activity towards alachlor and CDNB (*11, 13, 18, 21*) and isomerizes thiadiazolidin-one herbicides much better than GST I (*11, 19, 20*). GST IV is a safener-inducible (by benoxacor [4-(dichloroacetyl)-3,4-dihydro-3-methyl-2*H*-1,4-benzoxazine]) homodimer of two GST27 subunits with activity towards acetochlor, alachlor and metolachlor (*1, 22*). Homodimer GST III (two GST26 subunits) may be induced by cadmium (*23*) and dichlormid (*1*) and is active towards alachlor, metolachlor and CDNB (*1, 16, 17, 24*). A revised nomenclature (*25, 26*) was proposed for these GSTs based on their subunit composition with a prefix indicating the plant source *Zea mays* (*Zm*). Corn isoenzyme GST I corresponds to *Zm* GST I-I, GST II to *Zm* GST I-II, GST III to *Zm* GST III-III and GST IV to *Zm* GST II-II. *Zm* GST I-III is a constitutive heterodimer with activity against chloroacetamides and fluorodifen [2,4'-dinitro-4-trifluoromethyl diphenylether] (*26*). Homodimer *Zm* GST V-V is selectively inducible by the safener dichlormid (*25*) with activity towards diphenyl ether herbicides. Heterodimer *Zm* GST V-VI is an auxin-inducible isoform with activity against

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch014

metolachlor (*25*). BZ 2 is an inducible type III corn GST (26 kDa) with activity towards CDNB and some natural substrates (*27*).

Here we report the purification of four recombinant *N*-terminal 6xHis-tagged corn GST isoforms from *E. coli* and their biochemical characterization. Activities of these enzymes with endogenous, toxic alkenal substrate analogues, GST-mediated peroxidase activity as well as immunological detection of GST subunits were studied.

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch014

Materials and Methods

cDNA Cloning and Construction of Plasmids

Techniques of DNA manipulations were used as described (*28*). All PCR reactions were performed in a Programmable Thermal Controller PTC-100 (MJ Research Inc., Watertown, Mass., USA) using a standard program (*29*). Expression vectors pQE30 (amp^r; ampicillin resistance) and pQE31 (amp^r) were from Qiagen (Hilden, Germany). cDNAs of corn GST-subunits GST29 (*13, 14*), GST27 (*13, 15*), and GST26 (*16*), cloned in plasmids (pIJ12), (pIJ21) and (pGTC20), respectively, were used as templates for PCR. Primers for PCR were designed so that the entire coding regions of the cDNAs would be amplified and subsequently expressed in frame. Sequence analysis of DNA and proteins was performed with PC Gene program (version 9.0, 1993; IntelliGenetics, Geest, Belgium).

Plasmids pGST26, pGST27 and pGST29 are expression vectors of the GST26, GST27 and GST29 cDNA, respectively, and were constructed as described previously (*29*). Plasmid pGST27-29 (coexpression vector of the GST27 and GST29 cDNA (recombinant protein GST II)) was constructed to yield a single mRNA transcription product which includes the GST27 and GST29 coding sequences. Each GST subunit is assumed to be translated separately (since the mRNA includes two ribosomal binding sites for each coding sequence) resulting in 6xHis-tagged GST subunits GST29 and GST27. Transcription and translation of these subunits that are in close proximity should promote formation of heterodimeric GST II. Each plasmid was transformed by electroporation into *E. coli* M15 host strain (Qiagen, Hilden, Germany) containing the repressor plasmid pREP4 (kan^r; kanamycin resistance).

Expression of cDNAs and protein purification under native conditions

Transformed *E. coli* strains were grown and recombinant GST-isoforms were purified with nickel-nitrilotriacetic acid (Ni-NTA) agarose resin (Qiagen, Hilden, Germany) as detailed previously (*29*).

Purification of GST II from Corn

Isoform GST II was purified from safener-treated corn seedlings using the protocol described previously (*11*) until the step of glutathione Sepharose.

Polyacrylamide Gel Electrophoresis (PAGE)

Expression of the transformed *E. coli* strains as well as purity, molecular weight and quantitative amounts of recombiant GST isoforms were analyzed by 15%-SDS-PAGE according to Laemmli (*30*).

Oxyfluorfen-treated Plants

For Western blots, corn seedlings were cultivated as follows. Corn was sterilized for 20 min with 1.3% sodium hypochlorite, washed three times and sown into glass pots containing 80 ml substrate (Murashige and Skoog basal medium, Sigma, Deisenhofen; 0.9% (w/v) agar, Difco Lab., Detroit, USA; pH 5.8) under sterile conditions.

Corn seedlings were kept at 24°C with 16 h light per day [60 mmol photons m^{-2} s^{-1} (65 W, neon tubes "universal white" and "warm universal white", Osram, Munich, Germany)]. After three days they were transferred to a medium containing 500 nM oxyfluorfen and 0.1% (v/v) acetone and grown for an additional three days under the same conditions.

Homogenization of Oxyfluorfen-treated Corn Seedlings

Corn seedlings were separated from their roots, mixed with buffer (50 mM sodium phosphate, pH 6.8; 1 mM EDTA (ethylenediaminetetraacetic acid); 1 mM $MgCl_2$; 1 mM dithiothreitol; 10% (v/v) glycerol; 0.2% (w/v) polyvinylpyrrolidone, and 0.1 mM phenylmethylsulfonyl fluoride) and milled to a fine powder in a mortar with liquid nitrogen. The upper phase of a 10-min centrifugation at 4°C was diluted 1:1 (v/v) with SDS (sodium dodecylsulfate) PAGE sample buffer and boiled for 2 min.

Production of Antisera and Western-Blotting

Purified, recombinant homodimers GST I, III and IV were electro-eluted from SDS-PAGE. Antisera were raised in rabbits to recombinant GST26, GST27 and GST29 subunits, respectively.

After SDS-PAGE, proteins were electro-blotted (ref. *31*, with modification) onto Immobilon-P PVDF membrane (Millipore, Eschborn, Germany). PBS/Tween (4.1 mM NaH_2PO_4; 6.7 mM Na_2HPO_4; 140 mM NaCl and 0.05% (v/v) Tween-20) was used as washing buffer. Membranes were blocked (5% (w/v) low fat dry milk) and probed with one antiserum (antisera were preincubated overnight at 4°C with 5 µg of the other two GST homodimers).

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch014

Membranes were treated with secondary anti-rabbit IgG antibody coupled to alkaline phosphatase. Bound antibodies were visualised using 150 µg/ml p-nitrotetrazolium blue (NBT) and 25 µg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP; disodium salt) in glycine buffer (0.1 M glycine/NaOH, pH 9.5; 1 mM Mg- and 1 mM Zn-chloride).

Molecular Weight Calculation (Gel Filtration)

Native apparent molecular weights of recombinant GST isoforms were determined by gel filtration (*29*), which was performed on Superose 12 (HR 10/30; Pharmacia, Freiburg, Germany).

The concentration of protein was determined by the method of Bradford (*32*) using bovine serum albumin as protein standard.

Glutathione S-Transferase Assays

Conjugation of CDNB (1-chloro-2,4-dinitrobenzene; Sigma, Deisenhofen, Germany) and reduced glutathione (GSH) was measured spectrophotometrically (*11, 33*) at 340 nm (ε_{340} = 9.6 mM^{-1} cm^{-1}) and 30°C (29). Turnover of [^{14}C]-labelled metazachlor was performed as described (*11, 29*) at 35°C. Isomerization of thiadiazolidine BW78 [5-(4-bromophenylimino)-3,4-tetramethylene-1,3,4-thiadiazolidin-2-one] was carried out at 30°C as described (*11, 29*).

Conversion of ethacrynic acid (Sigma, Deisenhofen, Germany) was measured at 270 nm (*34*) (ε_{270} = 5 mM^{-1} cm^{-1}) and 30°C (*29*). Conjugation of crotonaldehyde (Fluka, Deisenhofen, Germany) was measured at 230 nm (*35*) (ε_{230} = 10.7 mM^{-1} cm^{-1}) and 30°C (*29*).

Glutathione Peroxidase Assays

Glutathione peroxidase activity (*36, 37*) was measured using a glutathione reductase coupled assay to monitor the oxidation of GSH. Consumption of NADPH at 30°C was monitored spectrophotometrically at 340 nm. Enzymic conversion of cumene hydroperoxide (Sigma, Deisenhofen, Germany) (*38*), *tert.*-butyl hydroperoxide (Sigma, Deisenhofen, Germany) (*37*) and linolenic acid hydroperoxide (*39*) was measured by a standard assay (*29*) [1 ml, performed in 50 mM K-phosphate buffer, pH 7.0, containing 2 mM GSH, 0.2 mM NADPH (Sigma, Deisenhofen, Germany), 2 mM EDTA, 0.5 units glutathione reductase and an organic hydroperoxide substrate (1.5 or 2 mM). Consumption of NADPH at 30°C was monitored spectrophotometrically at 340 nm].

Preparation of Linolenic Acid Hydroperoxide

Linolenic acid (9,12,15-octadecatrienoic acid) was converted enzymatically (*29*) to linolenic acid hydroperoxide (13-hydroperoxy-9,11,15-octadecatrienoic

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch014

acid) using soybean lipoxygenase (EC 1.13.11.12, 64,000 units/mg; Sigma, Deisenhofen, Germany).

Chemicals and Statistics

All chemicals used were of highest purity available. [Phenyl-U-^{14}C]metazachlor (11,6 mCi/mmol = 429 MBq/mmol) was a generous gift from BASF AG, Limburgerhof, Germany. Thiadiazolidine BW78 and triazolidine BW85 were from Dr. Y. Sato and Dr. K. Wakabayashi, Department of Agricultural Chemistry, Tamagawa University, Tokyo, Japan. Unless indicated otherwise, the results documented in the tables represent mean values with maximum standard deviation from three independent experiments.

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch014

Results and Discussion

Recombinant GST Isoforms

Expression of GST29, GST27 and GST26 cDNA resulted in accumulation of corresponding recombinant 6xHis-tagged GST subunits. Most of the recombinant protein was soluble and could be isolated in a batch purification with Ni-NTA agarose resin to electrophoretic homogeneity (Figure 1).

The co-expression of the GST27 and GST29 cDNA resulted in small amounts of the homodimer GST I, but much larger quantities of heterodimer GST II and homodimer GST IV, respectively (*29*). Seemingly, formation of heterodimer GST II is favoured, because almost no homodimer GST I was found, and formation of homodimer GST IV is due to excess expression of subunit GST27.

In Table I three combinations of homodimer mixtures are shown added together in a 1:1 molar ratio. No heterodimers 26/27 or 26/29 were formed from the homodimers present. There was, however, an almost complete 27/29 GST II-heterodimer formation when 27/27 and 29/29 homodimers were mixed. Furthermore, once the GST-II heterodimer has been formed, no back reaction to a homodimer was observed. Future studies should find out, whether modified conditions will produce 26/27- or 26/29 dimers. It should be noted, that heterodimer 26/27 has not yet been found in corn extracts.

All recombinant GST subunits carry a *N*-terminal 6xHis-tag resulting in a slight increase of the calculated molecular weights (GST29: 23.6 + 2.0 kDa; GST27: 24.4 + 1.6 kDa and GST26: 23.6 + 1.6 kDa). Calculated isoelectric points correspond to measured values of natural corn GST subunits [GST29: pI = 6.06 (*16*); GST27: pI = 6.1 (*15*); GST26: pI = 6.34 (*16*)].

Molecular weights of native isoforms were calculated from a linear fit of protein standards performed by gel filtration (data see ref. *29*). Each recombinant GST (molecular weights: GST I, 51 ± 2 kDa; GST II, 54 ± 4 kDa; GST IV, 56 ± 3 kDa and GST III, 48 ± 2 kDa) eluted as a single peak and the determined values of molecular weights were found as estimated for dimeric forms (*29*).

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch014

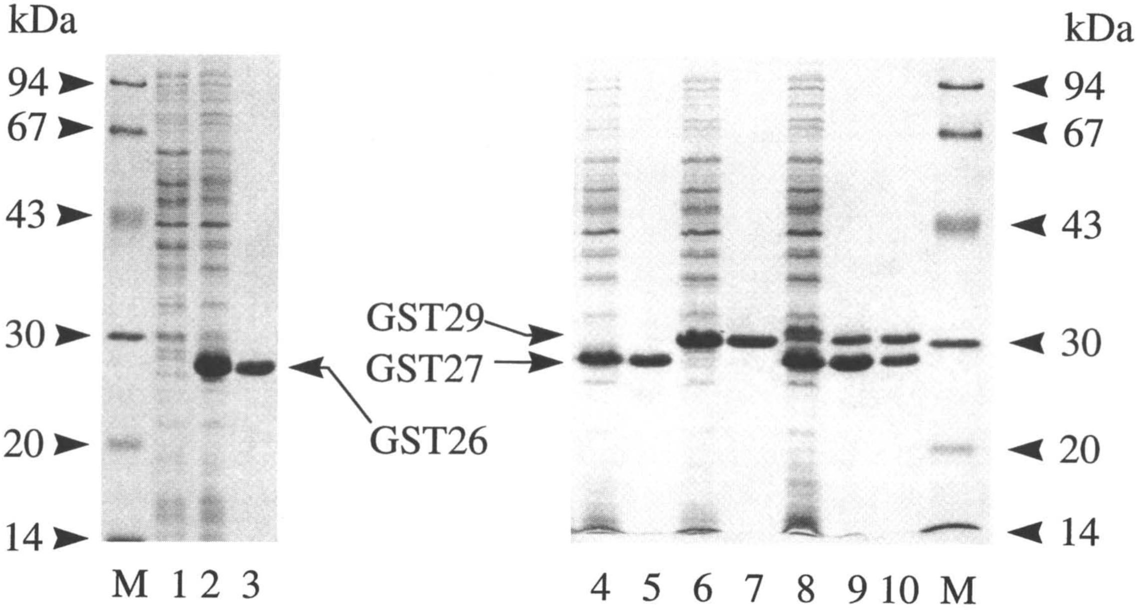


Figure 1. SDS-PAGE analysis of GST cDNA expression in E. coli and purification of recombinant corn GST isoforms. M: marker proteins with apparent molecular weights; 1: E. coli control; 2: expression of GST26 cDNA in E. coli; 3: purified 6xHis-tagged protein GST III; 4: expression of GST27 cDNA; 5: purified 6xHis-tagged GST IV; 6: expression of GST29 cDNA in E. coli; 7: purified 6xHis-tagged protein GST I; 8: coexpression of GST29 and GST27 cDNA; 9: purified 6xHis-tagged protein mixture from coexpression; 10: separated GST II heterodimer.

Table I. Formation of GST Heterodimers *in vitro*[a]

Homodimer Mixture	*Heterodimers Formed*
GST I (29/29) + GST III (26/26)	none detected
GST III (26/26) + GST IV (27/27)	none detected
GST I (29/29) + GST IV (27/27)	about 90% GST II (27/29); no reversion to homodimer

[a] Incubation for 24 h, 0°C. Aliquots of homodimers were mixed, 10 µg protein per 10 µl buffer solution. This one included 50 mM K-phosphate, pH 7.5, 1 mM EDTA, 10% glycerol, and 0.1% Tween-20.

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch014

Substrate Specificities of Recombinant GST Isoenzymes

The activities of the purified recombinant GST isoforms differed markedly (Table II). Using CDNB as substrate, the order of activity was GST I > GST II > GST III; and GST IV had no activity. Thus, both homo- and heterodimer GST isoforms, including subunit GST29, exhibit CDNB conjugation activity. Conjugation of metazachlor was catalyzed at high rates by GST II or GST IV and at low rates by GST I or GST III. No data for metazachlor activity of natural GST IV and GST III are available in the literature. Apparently, GST isoforms including subunit GST27 are active in metazachlor conversion.

GST I showed very low, GST III almost no isomerization of thiadiazolidine BW78 to its isomer triazolidine BW85. High isomerization rates were determined in assays with GST II and GST IV. GST isoforms with a GST27 subunit are also active in isomerization of the thiadiazolidine. No isomerase activity has been demonstrated by natural GST IV and GST III.

The above three substrates were also used previously to determine activities of natural corn GST I (conversion of CDNB: 4894 µmol/h/mg; metazachlor: 0.07 µmol/h/mg; thiadiazolidine BW78: 0.33 µmol/h/mg) and GST II (CDNB: 2642 µmol/h/mg; metazachlor: 5.40 µmol/h/mg; thiadiazolidine BW78: 13.20 µmol/h /mg) (*11*). Apart from minor variations, no significant difference in substrate specificity between recombinant and natural corn GST was detectable. Activities of corn GSTs towards CDNB available in literature, measured in slightly modified assays, are comparable with values in this study [natural GST I (*18, 26*; heterologous GST I (*40*); natural GST II (*18, 26*); natural GST IV was not detected by (*22*); heterologous GST III (*40*)].

Besides standard GST activities, two substrate analogues of *α,β*-unsaturated aldehydes were also used as GST substrates (Table II). Endogenous, toxic *α,β*-unsaturated aldehydes are produced as a result of free-radical-induced lipid peroxidation and react with cellular constituents including DNA (*35*). Crotonaldehyde is a substrate analogue of naturally occurring alkenals formed during the oxidation of fatty acids and nucleic acids (*35*). Ethacrynic acid (*34*), an *α,β*-unsaturated ketone, has been described as a substrate analogue and an inhibitor of GSTs (*41*).

Table II. Substrate Specificities of Purified Recombinant Corn GST Isoforms

Substrate	*Specific Activity [μmol/mg protein/ h]*			
	GST I	*GST II*	*GST IV*	*GST III*
Thiadiazolidine BW78	1.05^{a} ± 0.15	9.18 ± 0.45	11.91 ± 0.25	0.05 ± 0.01
CDNB	5120^{a} ± 80	3110 ± 230	120 ± 20	530 ± 10
$[^{14}C]$Metazachlor	0.44^{a} ± 0.05	5.77 ± 0.83	4.37 ± 0.65	0.34 ± 0.02
Crotonaldehyde	1.74^{b} ± 0.11	1.86 ± 0.19	3.46 ± 0.23	1.13 ± 0.23
Ethacrynic acid	22.74^{b} ± 1.92	29.28 ± 0.78	37.32 ± 0.96	18.96 ± 0.48

[a] Values represent the mean ± standard deviation (SD; n = 3).
[b] Values represent the mean ± standard deviation (SD; n = 5).

All four recombinant GST isoforms react with crotonaldehyde, with highest activity by GST IV. GST isoforms including subunit GST27 had the highest and GST III the lowest activity. No data for the corresponding natural GST isoenzymes are available in the literature.

Each recombinant GST isoenzyme was also active towards ethacrynic acid. Recombinant GST I and GST III showed similar activities, i.e., GST II exhibited more than the latter two and GST IV gave the highest conversion rate. Obviously, again the activity for this substrate is due to GST isoforms containing the GST27 subunit. Activities available in reference (*26*) for natural GST I and GST II were higher than in this study. Ethacrynic acid was the better substrate for GSTs compared to crotonaldehyde, when based on the maximum rate (V_{max}) to express specific activity.

Apparently, the additional *N*-terminal 6xHis-tag of recombinant GST isoenzymes has no significant influence on substrate specificities and activities. Accordingly, recombinant expression of GSTs in *E. coli* is a convenient system to provide sufficient amounts of pure GST isoforms for biochemical studies.

Glutathione Peroxidase Activities

Recombinant GST isoforms were checked for their ability to deactivate cytotoxic organic hydroperoxides. Endogenous products of oxidative damage initiated by hydroxyl radicals (*1*) were used to measure activities towards a naturally occurring unsaturated fatty acid hydroperoxide (linolenic acid hydroperoxide) and two model substrates, cumene hydroperoxide and *t*-butyl hydroperoxide.

Glutathione peroxidase activities of recombinant GST isoenzymes are shown in Table III. GST II and GST IV deactivated linolenic acid hydroperoxide with similar high rates and GST I or GST III had very low rates.

t-Butyl hydroperoxide was not a substrate for recombinant GST I and GST III. Activity of GST IV was twice as high as that of GST II. No data for the corresponding natural GSTs using these two substrates are available in the literature.

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch014

Table III. Glutathione Peroxidase Activities of Recombinant Corn GST Isoforms

Substrate	*Specific Activity [units^a /mg protein]*			
	GST I	*GST II*	*GST IV*	*GST III*
Linolenic acid hydroperoxide	$11.2^b \pm 0.3$	547.5 ± 18.2	553.8 ± 31.2	16.7 ± 0.4
t-Butyl-hydroperoxide	$0.02^b \pm 0.01$	1.81 ± 0.06	3.80 ± 0.08	0.04 ± 0.02
Cumene hydroperoxide	$2.9^b \pm 0.1$	222.8 ± 7.1	496.3 ± 28.4	9.4 ± 0.2

[a] 1 unit is defined as: 0.868 x [NADPH oxidized] /h / [GSH, initial] (*37*).

[b] Values represent the mean ± standard deviation (SD; n = 3).

Recombinant GST I showed almost no activity and GST III only low activity towards cumene hydroperoxide. The rate of conversion with GST IV was twice as high as with GST II. Data for natural GSTs (*26*) indicates no activity of GST I and activity of GST II (GST IV and GST III were not determined). We found that linolenic acid hydroperoxide and cumene hydroperoxide were better GST-substrates than *t*-butyl hydroperoxide. GST isoforms, including the safener-inducible GST27 subunit, showed greater catalytic efficiency in deactivation of organic hydroperoxides than constitutive GST isoforms I and III.

Discussion of Specific Activities

Crystal structures of GSTs have revealed kinetically independent binding sites (*2, 40*) in each GST subunit. So the average of activities of GST I and GST IV for each substrate should be expected when heterodimeric GST II is studied.

Surprisingly, specific activities of GST II shown in Table 1 do not follow this assumption with every substrate. While activity towards CDNB, ethacrynic acid, *t*-butyl hydroperoxide and cumene hydroperoxide seems to be additive, crotonaldehyde is converted with only half of the rate expected. In contrast, thiadiazolidine BW78 reacts almost 30% faster than expected, and metazachlor and linolenic acid hydroperoxide activities were double that of the anticipated values.

Probably, conformational shifts occur by interaction of subunits. This suggestion is supported by the observations of co-expression of subunits GST29 and GST27 in *E. coli.* Although a mixture of GST I, GST II and GST IV was expected, almost no GST I was found. Obviously, formation of GST II is energetically favoured under these conditions, and the remaining GST27 forms homodimer GST IV (see Table I).

Corn GSTs and Oxidative Stress

GSTs play a role in oxidative stress tolerance (*33*). As suggested (*26*), GSTs

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch014

should have an additional function in protecting plants from injury by cytotoxic agents formed during oxidative stress. When fatty acids are peroxidized, cell components such as enzymes, pigments and membranes may be damaged by free radicals derived from peroxidation processes (*42*). A new corn isoform *Zm* GST V-V (*25*) reportedly conjugates alkenals and deactivates cumene hydroperoxide at low rates, but does not sequester fatty acid hydroperoxides. GST-mediated glutathione peroxidase activity has also been reported for, e.g., pea (*43*) and *Arabidopsis thaliana* GSTs (*39*).

The role of GSTs is broader than anticipated some years ago. Firstly, these enzymes were thought to be instrumental as detoxifying enzymes catalyzing conjugation of herbicides and other phytotoxic compounds. Secondly, the catalytic isomerization of thiadiazolidine herbicides to their more effective peroxidizing triazolidine derivatives has to be added as a major activity exhibited by certain GST isoforms (*11*). Thirdly, peroxidase activities also have to be considered. Surprisingly, both bioactivation of thiadiazolidines and deactivation of toxic alkenals or organic hydroperoxides resulting from oxidative stress as reported in this study, is catalyzed at high rates by GST isoforms including subunit GST27. This subunit is the major inducible GST subunit in corn (based on both mRNA and protein level) (*13, 21*).

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch014

Immunological Studies

All recombinant GST subunits carry an *N*-terminal 6xHis-tag resulting in a slight increase of the apparent molecular weights (calculated increase: GST29: 23.6 + 2.0 kDa; GST27: 24.4 + 1.6 kDa and GST26: 23.6 + 1.6 kDa) on SDS-PAGE (Figure 2 A).

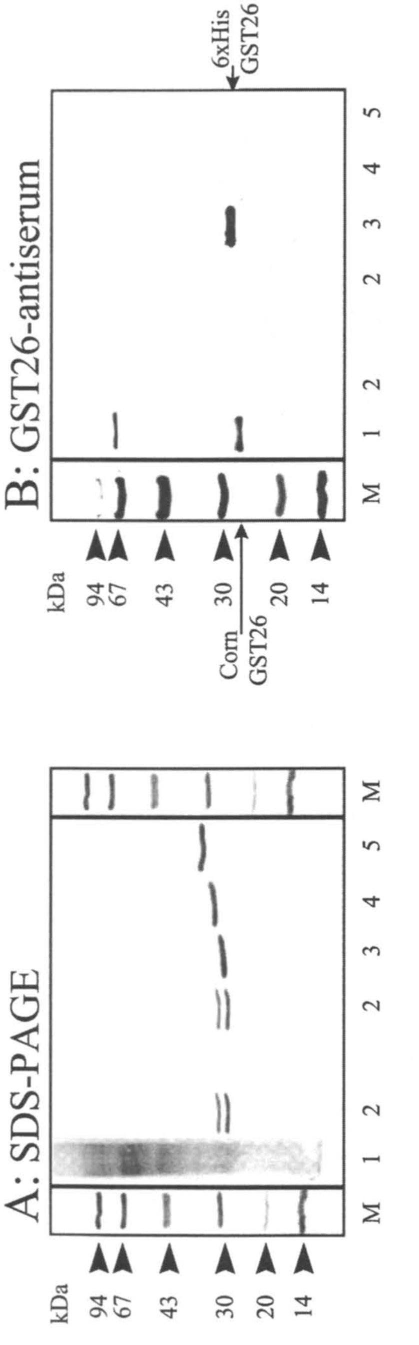
Using GST26-antiserum [diluted 1/30,000 (v/v)], Western blotting showed specific bands of correct molecular weight (Figure 2 B: recombinant GST26, lane 3 and crude corn extract, lane 1). No cross-reaction with GST27 or GST29 (recombinant lane 4,5; natural forms lanes 2) was observed. An additional band of crude corn extract (above 67 kDa) seems to be unspecific, because no GST subunit from corn with a molecular weight above 30 kDa is known.

Western blotting using GST27-antiserum [diluted 1/8,000 (v/v)] also showed bands of correct molecular weight (Figure 2 C: recombinant GST27 lane, 4, natural GST27 of purified corn GST II, lanes 2 (lower bands) and natural GST27 in crude corn extract (lane 1 lower band). Little cross-reaction was observed with the GST29 subunit (Figure 2 C: recombinant GST29, lane 5; natural GST29 of purified GST II, lanes 2 (upper bands) and in crude corn extract (upper band).

Using GST29-antiserum [diluted 1/20,000 (v/v)], Western blotting again showed specific bands of correct molecular weight (Figure 2 D: recombinant GST29, lane 5; natural GST29 of purified GST II from corn, lanes 2 and natural GST29 in crude corn extract, lane 1). No cross-reaction with GST26 or GST27 (recombinant lane 3,4; natural GST27 lanes 2) was observed.

In our experiment, GST27-antiserum cross-reacted with natural and recombinant GST29 subunits. In contrast, no cross-reaction was observed (*21*) using antiserum raised against GST27 for Western blotting. The GST27 and GST29 antisera were reported to complex, each specifically with their respective subunits. Corn GST27 shares 57% identity and 68.2% similarity (*13*) with the

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch014


A: SDS-PAGE
kDa
94
67
43
30
20
14
M
1
2
2
3
4
5
M
B: GST26-antiserum
kDa
94
67
43
30
20
14
Corn GST26
M
1
2
2
3
4
5
6xHis GST26

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch014

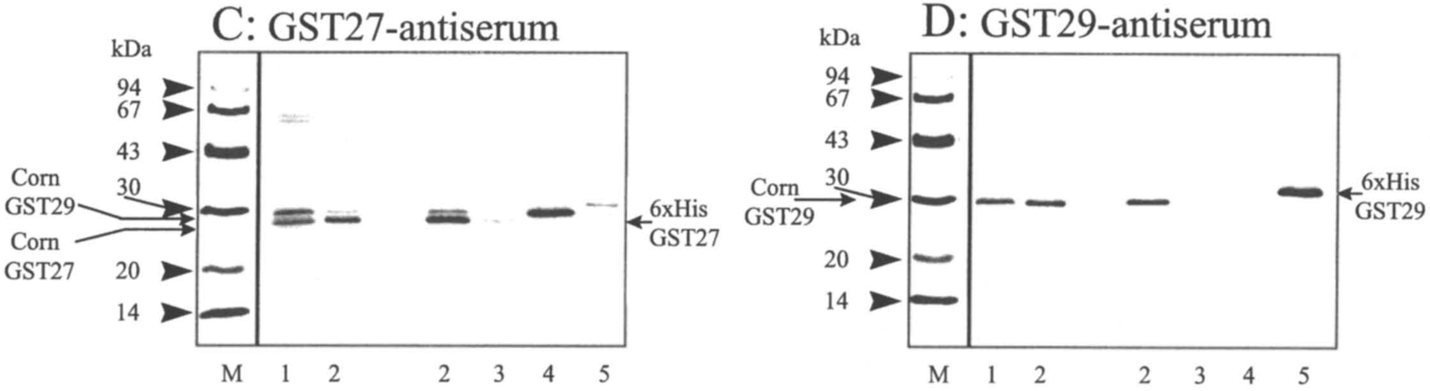

C: GST27-antiserum
kDa
94
67
43
30
20
14
Corn GST29
Corn GST27
6xHis GST27
M 1 2 2 3 4 5
D: GST29-antiserum
kDa
94
67
43
30
20
14
Corn GST29
6xHis GST29
M 1 2 2 3 4 5

Figure 2. Western-blots using GST26-, GST27- and GST29-antisera. M: stained marker proteins with apparent molacular weights; 1: crude protein extract from safener-treated corn seedlings; 2: purified GST II (GST27/GST29) from corn; 3: purified recombinant GST III (6xHis GST26); 4: purified recombinant GST IV (6xHis GST27); 5: purified recombinant GST I (6xHis GST29). A: SDS-PAGE of proteins; B: Western-blot using GST26-antiserum [diluted 1/30,000 (v/v)]; C: Western-blot using GST27-antiserum [diluted 1/8,000 (v/v)]; D: Western-blot using GST29-antiserum [diluted 1/20,000 (v/v)].

predicted amino acid sequence of the 29 kDa subunit of GST I and GST II. In our experiment, GST29-antiserum failed to cross-react with the GST27 subunit. Also no cross-reaction between GST27 and GST26 subunits (43.7% identity and 54.1% similarity (*13*)) or between the GST29 and GST26 subunits [50% identity (*40*)] was observed.

Acknowledgments

We are grateful to Prof. Dr. C.-P. Tu, Pennsylvania State University, University Park, PA, USA for providing plasmid pGTC20 and Dr. I. Jepson, ZENECA Seeds, Bracknell, UK for plasmids pIJ12 and pIJ21. Due thanks are expressed to Drs. K. Wakabayashi and Y. Sato, Department of Agricultural Chemistry, Tamagawa University, Tokyo, Japan, for a gift of thiadiazolidine BW78 and triazolidine BW85. This study was supported in part by the Fonds der Chemischen Industrie.

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch014

Literature Cited

1. Marrs, K. A. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1996,** *47*, 127-158.
2. Neuefeind, T.; Huber, R.; Reinemer, P.; Knäblein, J.; Prade, L.; Mann, K.; Bieseler, B. *J. Mol. Biol.* **1997,** *274*, 577-587.
3. Dixon, D. P.; Cummins, I.; Cole, D. J.; Edwards, R. *Current Opinion in Plant Biology* **1998,** *1*, 258-266.
4. Edwards, R.; Cole, D. J. *Pestic. Biochem. Physiol.* **1996,** *54*, 96-104.
5. Riechers, D. E.; Yang, K.; Irzyk, G. P.; Jones, S. S.; Fuerst, E. P. *Pestic. Biochem. Physiol.* **1996,** *56*, 88-101.
6. Cole, D. *J. Pestic. Sci.* **1994,** *42*, 209-222.
7. Hatton, P. J.; Cummins, I.; Cole, D. J.; Edwards, R. *Physiol. Plant.* **1999,** *105*, 9-16.
8. Timmerman, K. P. *Physiol. Plant.* **1989,** *77*, 465-471.
9. Knörzer, O. C.; Böger, P. In *Peroxidizing Herbicides;* P. Böger and K. Wakabayashi, Eds.; Springer-Verlag: Berlin, 1999, pp. 303-327.
10. Böger, P.; Wakabayashi, K. *Z. Naturforsch.* **1995,** *50c*, 159-166.
11. Nicolaus, B.; Sato, Y.; Wakabayashi, K.; Böger, P. *Z. Naturforsch.* **1996,** *51c*, 342-354.
12. Droog, F. *J. Plant Growth Regul.* **1997,** *16*, 95-107.
13. Jepson, I.; Lay, V. J.; Holt, D. C.; Bright, S. W. J.; Greenland, A. *J. Plant Mol. Biol.* **1994,** *26*, 1855-1866.
14. Shah, D. M.; Hironake, C. M.; Wiegand, R. C.; Harding, E. I.; Krivi, G. G.; Tiemeier, C. *Plant Mol. Biol.* **1986,** *6*, 203-211.
15. Irzyk, G. P.; Potter, S.; Ward, E.; Fuerst, E. P. *Plant Physiol.* **1995,** *107*, 311-312.
16. Grove, G.; Zarlengo, R. P.; Timmerman, K. P.; Li, N.; Tam, M. F.; Tu, C-P. D. *Nucl. Acid Res.* **1988,** *16*, 425-438.
17. Moore, R. E.; Davies, M. S.; O'Connell, K. M.; Harding, E. I.; Wiegand, R. C.; Tiemeier, D. C. *Nucl. Acid Res.* **1986,** *14*, 7227-7235.

18. Mozer, T. J.; Tiemeier, D. C.; Jaworski, E. G. *Biochemistry* **1983**, *22*, 1068-1072.
19. Iida, T.; Senoo, S.; Sato, Y.; Nicolaus, B.; Wakabayashi, K.; Böger, P. *Z. Naturforsch.* **1995**, *50c*, 186-192.
20. Sato, Y.; Böger, P.; Wakabayashi, K. *J. Pestic. Sci.* **1997**, *22*, 33-36.
21. Holt, D. C.; Lay, V. J.; Clarke, E. D.; Dinsmore, A.; Jepson, I.; Bright, S. W. J.; Greenland, A. J. *Planta* **1995**, *196*, 295-302.
22. Irzyk, G. P.; Fuerst, E. P. *Plant Physiol.* **1993**, *102*, 803-810.
23. Marrs, K. A.; Walbot, V. *Plant Physiol.* **1997**, *113*, 93-102.
24. O'Connell, K. M.; Breaux, E. J.; Fraley, R. T. *Plant Physiol.* **1988**, *86*, 359-363.
25. Dixon, D. P.; Cole, D. J.; Edwards, R. *Plant Mol. Biol.* **1998**, *36*, 75-87.
26. Dixon, D. P.; Cole, D. J.; Edwards, R. *Pestic. Sci.* **1997**, *50*, 72-82.
27. Marrs, K. A.; Alfenito, M. R.; Lloyd, A. M.; Walbot, V. *Nature* **1995**, *375*, 397-400.
28. Sambrook, J.; Fritsch, E. F.; Maniatis, T. Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory, New York, **1989**.
29. Sommer, A.; Böger, P. *Pestic. Biochem. Physiol.* **1999**, *63,* 127-138.
30. Laemmli, U. K. *Nature* **1970**, *227,* 680-685.
31. Towbin, H.; Staehelin, T.; Gordon, *J. Proc. Natl. Acad. Sci. USA* **1979**, *76*, 4350-4354.
32. Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248-254.
33. Knörzer, O. C.; Durner, J.; Böger, P. *Physiol. Plant.* **1996**, *97*, 388-396.
34. Habig, W. H.; Jakoby, W. B. *Methods Enzymol.* **1981**, *77*, 398-405.
35. Berhane, K.; Widersten, M.; Engström, Å.; Kozarich, J. W.; Mannervik, B. *Proc. Natl. Acad. Sci.* **1994**, *91*, 1480-1484.
36. Flohé, L.; Günzler, W. A. *Methods Enzymol.* **1984**, *105*, 114-121.
37. Wendel, A. *Methods Enzymol.* **1981**, *77*, 325-333.
38. Simmons, T. W.; Jamall, I. S.; Lockshin, R. A. *Comp. Biochem. Physiol.* **1989**, *94b*, 323-327.
39. Bartling, D.; Radzio, R.; Steiner, U.; Weiler, E. W. *Eur. J. Biochem.* **1993**, *216*, 579-586.
40. Neuefeind, T.; Huber, R.; Dasenbrock, H.; Prade, L.; Bieseler, B. *J. Mol. Biol.* **1997**, *274*, 446-453.
41. Droog, F. N. J.; Hooykaas, P. J. J.; van der Zaal, E. *J. Plant Physiol.* **1995**, *107*, 1139-1146.
42. Kunert, K. J.; Homrighausen, C.; Böhme, H.; Böger, P. *Weed Sci.* **1985**, *33*, 766-770.
43. Edwards, R. *Physiol. Plant.* **1996**, *98*, 594-604.

Downloaded by STANFORD UNIV GREEN LIBR on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch014

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch015

Chapter 15

Genetics of Atrazine and *s*-Triazine Degradation by *Psedomonas* sp. Strain ADP and Other Bacteria

Michael J. Sadowsky[1-3] and Lawrence P. Wackett[2-4]

[1]Department of Soil, Water, and Climate, [2]Biological Process Technology Institute, [3]Center for Biodegradation Research and Informatics, and [4]Department of Biochemistry, Molecular Biology, and Biophysics, University of Minnesota, St. Paul, MN 55108

Atrazine [6-chloro-N^2-ethyl-N^4-isopropylamino-1,3,5-triazine-2,4-diamine], one of the most widely used herbicides in the United States, is rapidly mineralized by *Pseudomonas* sp. strain ADP in soils and water. *Pseudomonas* strain ADP metabolizes atrazine as its sole source of nitrogen for growth and initiates atrazine catabolism via three enzymatic steps encoded by the genes *atzA*, *B* and *C*. The first enzyme, AtzA, catalyzes the hydrolytic dechlorination of atrazine yielding hydroxyatrazine. The second enzyme, AtzB, catalyzes hydroxyatrazine deamidation yielding *N*-isopropylammelide, and the third, AtzC expresses *N*-isopropylammelide isopropylamino hydrolase activity, metabolizing *N*-isopropylammelide stoichiometrically to cyanuric acid and *N*-isopropylamine. Cyanuric acid is subsequently metabolized to CO_2 and NH_3 via a lower pathway. We have cloned and sequenced the first three genes in the metabolic pathway and have over-expressed these proteins in *E. coli*. We have shown that genes homologous to *atzA*, *B* and *C* are present in different genera of atrazine-degrading bacteria isolated from geographically diverse locations of the U.S. and World. The *atzA*, *B* and *C* genes have been localized to a 97 kilobase, self-transmissible, plasmid, pADP-1,in *Pseudomonas* ADP and self-transmissibility of the plasmid was shown by its transfer to *Escherichia coli* and other bacteria. The *atzA* gene is flanked by DNA showing greater than 95% sequence identity to insertion sequence IS1071 from *Alcaligenes* and other bacteria harboring catabolic plasmids, suggesting that transposases may have played a role in the assembly of this metabolic pathway.

> Taken together these data indicate atrazine catabolism via hydroxyatrazine is widespread in nature and suggest a potential molecular mechanism for the global dispersion of the *atzA*, *atzB*, *atzC* genes.

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch015

Atrazine [6-chloro-N^2-ethyl-N^4-isopropylamino-1,3,5-triazine-2,4-diamine] (Figure 1) is a herbicide used to control of broad-leaf weeds and is a predominant member of a large class of symmetrical (s)-triazine herbicides. Atrazine has been extensively used to control weeds in corn, sorghum, sugarcane, and other crops. Other widely used *s*-triazine herbicides include simazine [(6-chloro-N^2,N^4-diethyl-1,3,5-triazine-2,4-diamine], used mostly to control weeds in tree fruit and nut orchards, and vineyards, and cyanazine [2-(4-chloro-6-ethylamino-1,3,5-triazin-2 -ylamino)-2-methyl-propiononitrile]. In the United States alone, about 800 million pounds of atrazine were used from 1980-1990 (*1*). Atrazine can persist in soils from months to weeks (*2,3*) and several studies have shown that atrazine transformation in certain environments proceeds relatively slowly (*4,5*). Atrazine and several of the *s*-triazine derivatives are relatively mobile in soil and this has led to the contamination of ground and surface water in several countries (*6-13*). Atrazine mobility in soils is directly influenced by its water solubility (33 mg kg^{-1}), moderate sorption coefficient (K_{oc} 160 mL g^{-1}) and biodegradation rate (*14*). Soil surface preparation, soil structure, initial water content, the presence of earthworm burrows, and time of application relative to rainfall events also influence the movement of atrazine in soils (*15*). Water runoff transport pathways and leaching in porous soils have led to the contamination of groundwater by atrazine (6,*7,15*). This has prompted many researchers to look for microorganisms that have the ability to degrade atrazine in soils and water. Atrazine and other s-triazine herbicides have been traditionally applied for broadleaf weed control in corn. While corn is tolerant to atrazine, simazine, and other s-triazine herbicides, many other agronomic species do not share this resistance. Several studies have shown that atrazine and simazine residues in soil cause injury to crops in subsequent growing seasons (*16-23*). Atrazine is taken-up from soil and translocated to plant leaves (*24*). Plant injury is usually observed first in young seedlings as marginal chlorosis on lower leaves and slowly advances down the leaf and up the plant (*19*). Residue problems associated with the use of triazine herbicides have been reported in most corn growing areas of North America (*19,22,23*).

Atrazine Degradation

The removal of atrazine from soils is limited by both biotic and abiotic factors. Several environmental factors, including soil pH (*24,25*), temperature (*26*), and moisture (*27*), sorption (*28*), tillage practices (*29*) and several other soil properties (*30*) have been shown to influence atrazine persistence in soils. In plants such as corn, transformation of atrazine, simazine, and propazine is initiated by one of three, often competing reactions involving: *N*-dealkylation (via P-450 monooxygenases) of the side chains, hydrolytic dehalogenation, or displacement of the chloro group with gluathione (*31*). In the later case, atrazine and related s-triazine herbicides are metabolized in leaves of tolerant plants by the action of glutathione S-transferase (*31-33*). These reactions can result in total or partial loss of phytotoxicity. Dechlorination of *s*-triazines by microbial gluathione conjugation systems has not been observed (*34*).

There is currently little debate that microorganisms (*7,35-46*) degrade atrazine in soil. Until relatively recently, however, there have been few reports of the isolation of pure cultures of soil microorganisms that mineralize atrazine. In bacteria, biodegradation of s-triazine compounds, including atrazine, can occur by *N*-dealkylation and dechlorination processes (*38*). Generally speaking, *s*-triazine compounds lacking bulky side group substituents are most likely degraded relatively rapidly in soils, due to bacterial-mediated dechlorination reactions (*47,48*). An enzyme from ***Rhodococcus corallinus*** has been identified which catalyzes dechlorination of some chloro-*s*-triazine compounds, but it is inactive with atrazine (*42*). While dealkylation reactions have been suggested to be the first metabolic step in the biodegradation of atrazine (*38,44,49-51*), more recently, biological dechlorination of atrazine has also been shown to occur and be widespread in nature (*36,37,45,46*). For example, ***Pseudomonas*** sp. strain ADP rapidly dechlorinates atrazine to hydroxyatrazine (2-hydroxy-4-ethylamino-6-isopropylamino-*s*-triazine) (Figure 2) by an enzymatic hydrolytic reaction, mediated by an atrazine chlorohydrolase (*37,40,52*). Based on these studies, it is apparent that microorganisms have developed several biochemical mechanisms for degrading atrazine and related *s*-triazine compounds.

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch015

Atrazine-Degrading Bacteria

There have been numerous reports on the occurrence and isolation of *s*-triazine-degrading microorganisms (*7,35,38-44, 47,49,51,53-56*). A majority of the organisms described, however, failed to mineralize atrazine (*35,47*). While earlier studies reported atrazine degradation only by mixed microbial consortia, more recent reports indicate that several isolated bacterial strains can degrade and mineralize atrazine (Table 1). Mandelbaum *et al.* (*7*) reported the isolation of a pure bacterial culture, identified as ***Pseudomonas*** sp. strain ADP, which degraded a high concentration of atrazine (>1,000 μg mL^{-1}) under growth and non-growth conditions. ***Pseudomonas*** sp. strain ADP used atrazine as a sole source of nitrogen for growth and the organism completely mineralized the *s*-triazine ring of atrazine. About 80% of the added atrazine was degraded within 15 h of incubation and 100% was mineralized by 25 h. Radosevich *et al.* (*51*) also reported the isolation of a pure bacterial culture which degraded atrazine. This strain M91-3, which was subsequently identified as a ***Ralstonia*** sp. strain, used atrazine as a sole N or C source for growth and mineralized between 40 and 50% of added atrazine. Cell growth, however, was very moderate and there was limited growth (no change in absorbance) associated with atrazine use. In addition, Yanze-Kontchou and Gschwind (*44*) isolated a ***Pseudomonas*** strain, YAYA6, that partially mineralized atrazine during a 50-day incubation period. More recently, Bouquard *et al.* (*46*) reported that a ***Rhizobium*** sp. strain, PATR, has the

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch015

Table I. Some *s*-Triazine Degrading Bacteria and Their Substrates and Degradation Products

Microbe	Substrate (s)	Degradation Product(s)	Citation
Pseudomonas ADP	Atrazine, Simazine, Terbuthylazine	CO_2	7
Rhodococcus TE1	Atrazine, Simazine	Deisopropylatrazine Deethylatrazine	56
Pseudomonas 12227	Melamine	CO_2	47
Klebsiella pneumoniae	Ammelide	CO_2	47
Rhodococcus corralinus 15444	Dethylsimazine	*N*-ethylammelide	85
Klebsiella terragena DRS-1	Melamine	CO_2	101
Pseudomonas 12228	Ammeline	CO_2	47
Ralstonia M91-3	Atrazine	CO_2	51
Agrobacterium J14a	Atrazine	CO_2	41
Rhizobium PATR	Atrazine	CO_2	46
Alcaligenes SG1	Atrazine	CO_2	45
Bacterium 38/38	Atrazine	CO_2	69
Clavibacter michiganese ATZ1	Atrazine	*N*-ethylammelide	36
Pseudomonas CN1	*N*-ethylammelide	CO_2	36

ability to metabolized atrazine to hydroxyatrazine, via a dechlorination reaction, and Struthers *et al.* (*41*) and Boundy-Mills *et al.* (*45*) reported the isolation of strains of *Agrobacterium* and *Alcaligenes*, respectively, that mineralized atrazine. Pure cultures of atrazine-degrading bacteria have recently been used to investigate the biochemistry and genetics of atrazine catabolism in mixed microbial consortia (*36*).

Genetics of Atrazine and *s*-Triazine Degradation

Most of the current information concerning the genes and enzymes involved in the metabolism of atrazine is limited to our understanding of atrazine biodegradation in *Pseudomonas* sp. strain ADP. However, previous studies have shed some light on the genetics and biochemistry of the degradation of related *s*-triazine herbicides. An inducible set of genes that encode the enzymes for melamine (1,3,5- triazine-2,4,6-triamine) metabolism were isolated from *Pseudomonas* sp. strain NRRL B-12227 (*57,58*). While this strain did not metabolize atrazine, it degraded melamine in a six step pathway which liberates ammonia to support growth. Strain NRRLB-12227 also metabolized *N*-isopropylammelide, *N*-ethylammeline, ammelide, and cyanuric acid. Three of the genes involved in the melamine degradation pathway, *trzB*, *trzC*, and *trzD*, have been cloned. Similar degradation genes have been isolated from *Pseudomonas* sp. strain NRRL B-12228 and *Klebsiella pneumoniae* strain 99 (*57,58*). More recently, it has been shown that the genes encoding ammelide aminohydrolase (*trzC*) and cyanuric acid amidohydrolase (*trzD*), from strain NRRLB 12227, are located on a large IncI plasmid in *Klebsiella pneumonia* strain 99 (*59*). Moreover, the cyanuric acid amidohydrolase, *trzD*, from *Pseudomonas* sp. NRRL B-12227 has been sequenced (*60*).

Genes encoding atrazine degradation activity from *Rhodococcus* sp. strains have been reported (*43,61,62*). In *Rhodococcus* sp. strain TE1, *N*-dealkylation of atrazine is mediated by a single gene, *atrA* (*56*). *R. corallinus* NRRL B-15444R has the ability to dechlorinate the *s*-triazines desethylsimazine and desethylatrazine (*42*). The strain, however, does not metabolize atrazine or simazine. The gene responsible for the dechlorination/deamination has been sequenced and is termed *trzA* (*63*). A *Rhodococcus* cytochrome P-450 multicomponent monooxygenase system, encoded for by the *thcBCD* genes in *Rhodococcus* sp. strain NI86/21, catalyzes the *N*-dealkylation of atrazine to desethylsimazine and desethylatrazine (*64*). A recombinant *Rhodococcus* strain containing *atrA* and *trzA* catalyzes the multistep degradation of atrazine, but not its complete mineralization (*63*).

Genetics and Biochemistry of Atrazine Degradation in *Pseudomonas* sp. strain ADP

We are studying the genetics and biochemistry of microbial-mediated atrazine biodegradation to: 1) dissect the underlying biochemistry involved in atrazine degradation, 2) gain an understanding of the evolution of "recently evolved" microbial pathways, 3) produce gene probes for ecological analyses, for the

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch015

production of enzymes for bioremediation purposes, and 4) aid in the construction of superior biodegradative microorganisms and plants.

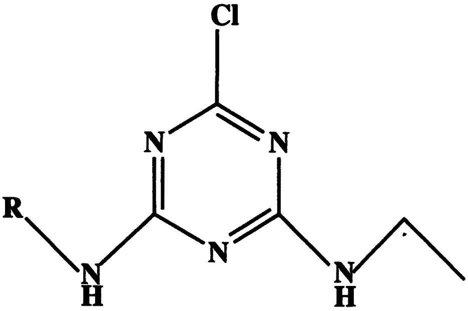
We have used a complementation approach to isolate and characterize gene regions in *Pseudomonas* sp. strain ADP encoding atrazine degradation activity (*37,45,52,65*). Our approach has been to clone and express atrazine-metabolizing genes from *Pseudomonas* sp. ADP in *E. coli* and delineate the genes responsible for catabolism by subsequent subcloning and *Tn*5 mutagenesis analyses. The first step was the construction and screening of a *Pseudomonas* sp. ADP total genomic library. This research was facilitated by the observation that *E. coli* clones containing the gene encoding the first metabolic step in the pathway, and potentially subsequent steps, produced clearing zones on agar plates containing 500 µg mL^{-1} atrazine, which is well above the 30 µg mL^{-1} solubility limit for atrazine in water (*7*). Previously, plates containing atrazine at a concentration exceeding its solubility limit had facilitated the isolation of *Pseudomonas* sp. ADP in pure culture (*37*). A 21.5-kilobase *Eco*RI genomic DNA fragment from *Pseudomonas* sp. strain ADP, designated pMD1, was shown to encode atrazine degradation activity in *E. coli.* A gene conferring the atrazine clearing phenotype was subsequently subcloned as a 1.9 kb *Ava*I fragment in pACYC184, designated pMD4, and was expressed in *E. coli* (*37*). Cloning and random Tn*5* mutagenesis showed that the 1.9 kb *Ava*I fragment was essential for atrazine dechlorination. Sequence data for the pMD4 gene region encoding atrazine transformation ability indicated that a single open reading frame of 1419 nucleotides, *atzA*, encodes atrazine dechlorination activity. Subsequent HPLC analyses indicated that *atzA* encodes atrazine chlorohydrolase that transforms atrazine to hydroxyatrazine (Figure 2). More recently, we showed that the *atzA* gene in *Pseudomonas* sp. strain ADP is flanked by DNA showing greater than 95% sequence identity to insertion sequence IS1071 from *Alcaligenes* sp. strain BR60 (*66*). Consequently, *atzA* is an ideal candidate for use in engineering bacteria and plants to metabolize atrazine to hydroxyatrazine, thereby remediating herbicide-containing soils.

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch015

Atrazine chlorohydrolase (AtzA) has been over-expressed in *E. coli* (pMD4) and purified using precipitation with 20% (w/v) NH_4SO_4 and anion exchange chromatography (*37*). The molecular weight of the holoenzyme is estimated by gel filtration chromatography to be 245,000. These results, combined with the deduced subunit molecular weight of 52,421 obtained via gene sequencing, is consistent with either an $\alpha4$ or $\alpha5$ subunit stoichiometry. The protein is activated by the addition of $CoSO_4$, $MnSO_4$, or $FeSO_4$ to assay mixtures (M. de Souza, University of Minnesota, unpublished). Currently, work is ongoing to determine if a specific coordination environment for metals exists and the role of such a putative site in catalysis.

The reaction catalyzed by AtzA is now understood in some detail. First, the conversion of atrazine to hydroxyatrazine is a hydrolytic reaction, as demonstrated by showing incorporation of $[^{18}O]$ from $[^{18}O]$-H_2O into the hydroxyl group of the product (*52*). Substrate specificity studies show that only substrates containing a chlorine atom, a fluorine atom, and a alkylamino side chain were hydrolyzed (*52,67*). These include atrazine, simazine, and terbutylazine. Melamine is not a substrate for AtzA. The K_m for atrazine is estimated to be 150 µM, the V_{max} is 2.6 µmol min^{-1} mg^{-1} protein, and the k_{cat} is 11 s^{-1}. These values are only approximate as both

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch015


Cl
N
N
N
R
N
H
N
H

Chemical Name	R Group
Atrazine	Isopropyl
Simazine	Ethyl
Cyanazine	Cyano-t-butyl

Figure 1. Structures of several s-triazine compounds degraded by bacteria.

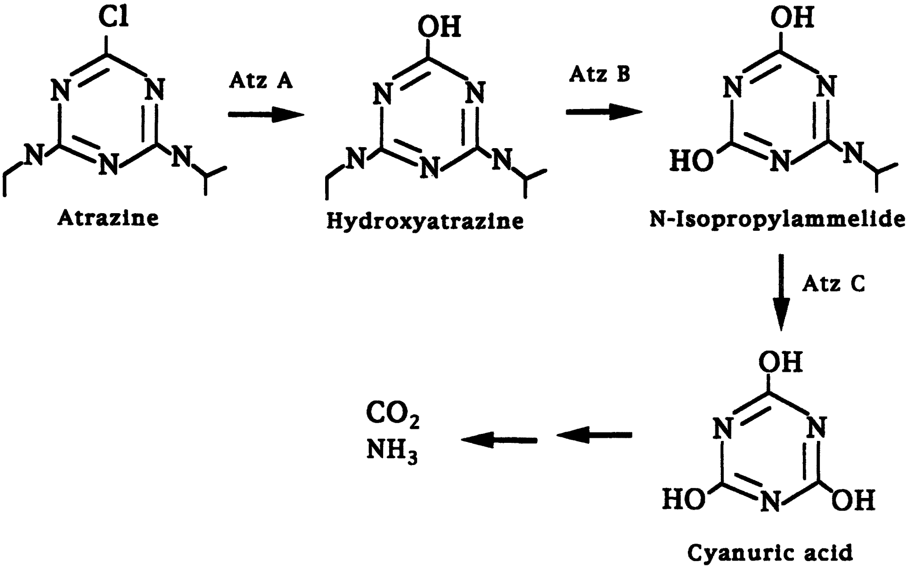

Atrazine
Atz A
Hydroxyatrazine
Atz B
N-Isopropylammelide
Atz C
Cyanuric acid
CO2
NH3

Figure 2. Metabolic pathway by which Pseudomonas strain ADP mineralizes atrazine to CO_2 and ammonia.

substrate and product are relatively insoluble. While this indicates that the activity of the natural enzyme for the herbicide is reasonably high, it can be possibly further improved by protein engineering. Such improvement may be significant for applications such as enzymatic waste water treatment or ground water decontamination.

Studies on the properties of AtzA are relevant to potential applications of this enzyme for the removal of atrazine from soil and drinking water supplies. Hydroxyatrazine is non-phytotoxic and has no demonstrable toxicity or carcinogenicity to mammals. In this context, the first metabolic step carried out by *Pseudomonas* sp. ADP represents the most advantageous situation from an environmental remediation standpoint. While the intact organism catalyzes atrazine hydrolysis and subsequent reactions, the enzyme could prove more efficacious for the following reasons: 1) *Pseudomonas* sp. ADP makes only a low level of AtzA because cell nitrogen needs are modest, 2) AtzA expression is down-regulated by inorganic nitrogen sources that are present in most contaminated soil and water, and 3) enzyme can be produced cheaply and in large quantity by recombinant bacteria. There are no regulatory concerns in adding a small amount of proteinaceous, non-living material that effectively catalyzes the hydrolysis of a highly regulated compound (atrazine) to a non-regulated compound (hydroxyatrazine). Studies are ongoing to explore the use of highly active AtzA in contaminated water remediation.

Transposon Tn*5* mutagenesis and subcloning studies were subsequently used to localized *atzB*, the second gene in the catabolic pathway. The *atzB* is located about 8.5 kb downstream from *atz*A on the same 21.5-kb genomic DNA fragment (pMD1) as *atzA*. The *atzB* gene encodes a 481 amino acid polypeptide that transforms hydroxyatrazine to *N*-isopropylammelide [2,4-dihydroxy-6 -(isopropyl amino)- *s*-triazine] (*45*). This is due to the apparent hydrolytic removal of the *N*-ethyl group of hydroxyatrazine. This enzyme, hydroxyatrazine aminohydrolase, cleaves the ethylamine side chain from the triazine ring, but does not remove the bulkier isopropylamine group. The AtzB had 25% amino acid identity with TrzA from *Rhodococcus corallinus*. Interestingly, over 600 nucleotides of upstream sequence were the same in *atzA* and *atzB* (*45*).

We have subsequently used *N*-isopropylammelide as the starting substrate to screen the *Pseudomonas* sp. strain ADP gene library for the third gene in the pathway, *atzC* (*65*). Several *E. coli* clones catalyzing the degradation of *N*-isopropylammelide to cyanuric acid were identified. Subsequent HPLC and GS-MS analyses confirmed that the product produced was cyanuric acid (Figure 2). The *atzC* gene was further delimited by functional analysis following transposon *Tn5* mutagenesis and subcloned as a 2.0 kb *Eco*RI-*Ava*I fragment. An *E. coli* strain containing this DNA fragment expressed *N*-isopropylammelide isopropylamino hydrolase activity, metabolizing *N*-isopropylammelide stoichiometrically to cyanuric acid and *N*-isopropylamine. The 2.0 kb DNA fragment was sequenced and found to contain a single open reading frame, *atzC*, of 1209 nucleotides, encoding a protein of 403 amino acids. Based on sequence comparisons, AtzC has been assigned to the amidohydrolase protein family that includes cytosine deaminase, urease, adenine deaminase, and phosphotriester hydrolase. Sequence comparisons in the most highly conserved regions indicated that AtzA and AtzB proteins also belong to the same amidohydrolase family (*65*).

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch015

The overall metabolic logic by which *Pseudomonas* ADP metabolizes atrazine is shown in Figure 2. We, at this time, have not isolated and characterized the genes and proteins in the lower pathway responsible for catalysis of cyanuric acid to CO_2. These genes, however, are most likely not unique to *Pseudomonas* ADP and other atrazine degraders, since several soil bacteria have been shown to catabolize cyanuric acid (*5,21,35,47,54*).

We have recently reported that genes homologous to *atzA*, *atzB* and *atzC* are present in five other recently identified atrazine-degrading bacteria, belonging to different genera and isolated from geographically diverse locations (*68,69*). These bacteria include: *Alcaligenes* sp. SG1 (from Louisiana), *Ralstonia* M91-3 (from Ohio), *Clavibacter michiganese* ATZ1 (from California), *Agrobacterium* J14a (from Nebraska), and an unknown bacterium 38/38 (from Indiana). Moreover, sequence analyses indicated that the *atzA*, *atzB*, and *atzC* genes in all five strains were greater than 99% identical, suggesting that horizontal transfer of atrazine degradation genes may have occurred recently.

The mechanisms by which the *atzA*, *B* and *C* genes could have possibly transferred amongst bacteria was revealed by molecular analysis. The *atz A,B and C* genes were localized to a 97-kilobase self-transmissible plasmid, pADP-1, in *Pseudomonas* sp. strain ADP. Self-transmissibility of pADP-1 was shown by its transfer to *Escherichia coli* and subsequent transfer to other *E. coli* strains (*68*) . The ability of this region of DNA to move selectively was observed in a derivative strain of *Pseudomonas* sp. ADP that showed a spontaneous loss of the *atzA* gene, a change in insertion element profile, and the retention of the *atzB* and *atzC* genes (*68*). The phenotypic instability noted in *Pseudomonas* sp. ADP, as seen for *atzA,B*, and *C* and *atzA* singly, has been observed with other catabolic pathways in numerous soil bacteria (*70-73*). In a parallel study, Topp *et al.* (*74*) characterized a number of atrazine-degrading bacteria from agricultural soil and found that all the isolates metabolized atrazine through hydroxyatrazine as an intermediate. In these isolates, several different plasmid patterns were observed. However, one plasmid of approximately 97-kb was common to all the atrazine catabolizing bacteria. The relationship of this plasmid with pADP-1 remains to be determined. An analogous plasmid-borne catabolic pathway in *Klebsiella pneumoniae* strain 99 (*75*) has been reported to metabolize some *s*-triazine compounds, but not atrazine. In that strain, the *trz*C, *D* and *E* genes encode for ammelide aminohydrolase, cyanuric acid aminohydrolase and biuret aminohydrolase, respectively, and are located on a 113 kb plasmid (*59,76*).

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch015

Evolutionary Aspects of Microbial Degradation of Triazine Herbicides

While chlorinated symmetrical triazines are the most widely used triazine herbicides, they have proven to be relatively recalcitrant in the environment. This difficulty was largely attributed to the fact that they are xenobiotic compounds, previously unexposed to microbial degradation activity (*77*). Whereas a few asymmetrical triazines are known to occur naturally [e.g. the antibiotic fervenulin (*78*)] and might have been previously metabolized by microorganisms, the *s*-triazines (symmetrical triazines) are generally regarded as xenobiotic (*33*). Microorganisms have succeeded in evolving catabolic pathways for the complete mineralization of chlorinated *s*-triazines, despite the fact that the ring carbon atoms are not sued as an energy source. There are many lines of evidence that suggest that microbial adaptation for the mineralization of *s*-triazine herbicides occurred over the time since their first introduction into agriculture in the mid 50's;

a) In most cases the half lives of *s*-triazines in soils with a history of *s*-triazine application are considerably shorter than in non-history soils (*79,80*);
b) Most of the microorganisms that were isolated for their ability to degrade *s*-triazines were isolated from soils with extensive exposure to s-triazines;
c) *s*-triazines that were considered non-biodegradable in the early years became biodegradable in later years. For example: melamine (triamine *s*-triazine) was considered non-biodegradable in the 30's (*81*), but in the 60's it was considered moderately biodegradable (*82*) and in 1981 it was reported to be completely biodegradable and registered as a slow release N fertilizer (*81,83,84*);
d) Over 200 bacterial colonies were isolated from an atrazine mineralizing mixed culture. None were found to individually degrade atrazine; however, when mixed together, the degradation ability was restored (*39*);
e) Extensive enrichment pressure was needed before pure cultures that were capable of atrazine mineralization were isolated from soil [e.g. *Pseudomonas* sp. strain ADP (*7*)]. The possibility for horizontal gene transfer during the enrichments is under current study (Sadowsky, unpublished);
f) For many years the complete mineralization of atrazine by bacteria was considered to be possible only by the joint efforts of two or more bacteria (consortia). For example in 1986 Behki and Khan (*49*) indicated that isopropyl removal from atrazine by *Pseudomonas* sp can supply substrate for complete mineralization by previously isolated rhodococci that could mineralize deisopropylatrazine ;
g) Despite extensive efforts over many years (*85*), only in 1993-1994 was a bacterium first isolated that could rapidly mineralize atrazine in pure culture (*86*). Interestingly, in a short time, several other pure bacterial cultures that could mineralize atrazine have been described (*41-44,51*);
h) The *N*-alkyl side chain of atrazine was considered to hinder bacterial dechlorination in the past (*49*), but in recent years bacterial mixed and pure cultures that could rapidly dechlorinate atrazine were isolated (*7,39,41,51*). Moreover, the bacterial gene responsible for the dechlorination (*52*); and the atrazine chlorohydrolase enzymes were characterized (*46,52*); and
i) Several microorganisms share near identical genes encoding the enzymes ammelide aminohydrolase and cyanuric acid amidohydrolase (*58,59*). This suggests that gene transfer between species plays an important role in the evolution and spread of *s*-triazine degradative capabilities within the soil microbial community (*59*).

Several insights into the molecular basis of atrazine metabolism further

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch015

strengthen our argument for the recent evolution of the *atzAB, and C* gene clusters on the catabolic plasmid, pADP-1. First, near simultaneous isolation of numerous atrazine-catabolizing bacteria by different research groups, after a history of failed efforts, is consistent with a recent appearance of pure cultures with this phenotype. Second, all the isolates contain the same suite of genes, but all nine non-atrazine-degrading bacteria did not contain *atzABC* homologs (*69*). Third, the atrazine plasmid is highly transmissible and thus could potentially spread to the diverse genera observed to contain the genes. Fourth, the genes are not contiguous and do not appear to be regulated at the gene level in response to the catabolite [M. de Souza, Unpublished], consistent with a sub-optimal gene arrangement. Fifth, the presence of the insertion elements provide a possible mechanism for the plasmid's construction and/or dispersal of the *atzA*, B and C genes. Insertion sequence elements and transposons are thought to be important in bringing about rapid evolutionary changes and many examples are known for catabolic pathways (*87-100*). Another observation consistent with the idea that atrazine genes are independently recruited in transposable cassettes is the recent observation of a *Rhizobium* species that metabolizes atrazine predominantly to hydroxyatrazine (*46*). This bacterium contains *atzA*, but does not contain *atzB* and *atzC*. The central portion of the *Rhizobium* atrazine chlorohydrolase had 22 out of 24 amino acids identical with the same AtzA region from *Pseudomonas* sp. ADP (M. de Souza, unpublished, University of Minnesota). This sequence identity is much greater than that possible by chance, Lastly, while the *atzA* and *atzB* genes have mol % G+C content of 58.3% and 64.1%, respectively, *atzC* has a 39.5% mol % G+C content, suggesting that the later gene was recruited from a different bacterial genus (*65*).

In summary, the ability to degrade atrazine appears to relatively widespread among several genetically and geographically unrelated bacteria. In all the cases studied by us, the microorganisms tested initiated atrazine degradation via atrazine chlorohydrolase, encoded by the *atzA* gene. Interestingly, the *atzA* gene from these diverse bacteria shared greater than 99% nucleotide identity with *atzA* from *Pseudomonas* ADP (*68,69*). This suggests that *atzA* in the different microbial genera was derived from a common ancestor that has diverged only to a limited extent. In addition, many of the organisms examined also contained the *atzB*, and *C* genes, suggesting that atrazine degradation by these disparate organisms occurs via a common mechanism. In light of these data, further work needs to be done to determine the assembly, transfer, and evolutionary history of these atrazine biodegradation genes that appear to be the predominant globally-distributed catabolic genes for the bacterial metabolism of this herbicide.

Acknowledgements

This work was supported, in part, by a grant from Novartis Crop Protection, by grant 94-34339-1122 from the United States Department of Agriculture-BARD program, and by grant 98-35107-6368 from the United States Department of Agriculture -NRI. We thank Jennifer Seffernick and Betsy Martinez-Vas for help with graphics.

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch015

Literature Cited

1. Gianessi, L. P. *Resources* **1987**, *89*, 1-4.
2. Anderson, P. C.; Georgeson, M. *Genome* **1989**, *31*, 994-999.
3. Cohen, S.; Creager, S.; Carsel, R.; Enfierld, C. *Treatment and Disposal of Pesticide Waste;* Krueger, R. F.; Seiber, J. N., Eds.; American Chemical Society: Washington, D.C., 1984; pp. 297-325.
4. Baker, F. W. G. In *Tropical Grassy Weeds*; Baker, F. W; Terry, P. J., Eds.; CAB International: Wallingford, England, 1991; pp. 96-105.
5. Erickson, E. L.; Lee, K. H. *Crit. Rev. Environ. Contam.* **1989**, *19*, 1-3.
6. Belluck, D. A.; Benjamin, S. L.; Dawson, T. In *Pesticide Transformation Products: Fate and Significance in the Environment*; Somasundaram, L.; Coats, J. R., Eds.; American Chemical Society: Washington, D.C., 1991; pp. 254-273.
7. Mandelbaum, R. T.; Allan, D. L.; Wackett, L. P. *Appl. Environ. Microbiol.* **1995**, *61*, 1451-1457.
8. Baker, B. D.; Richards, P. R. Lehr, J. H., Ed.; *Rational Readings on Environmental Concerns.* Van-Nostrand Reinhold, New York, N.Y. 1992; pp. 84-97.
9. Eisler, R. Atrazine Hazards to Fish, Wildlife, and Invertebrates. A Synoptic Review; Contaminant Hazard Review Report No. 18; Fish and Wildlife Service, U.S. Department of the Interior, Washington, DC., 1989.
10. Kello, D. *Food Add.& Contam.* **1989,** 6 Suppl. 1:S79-85.
11. Koplin, D. W.; Kalkhoh, S. J. *Environ. Sci. Technol.* **1993,** *27*, 134-139.
12. Parsons, D. W.; Witt, J. W. *Pesticides in Ground Water in the Unites States of America; a Report of a 1988 Survey of State Lead Agencies;* Oregon State Univ. Agri. Chem. Dept., Corvalis, OR, 1988.
13. Pick, F. E.; van Dyk, L. P; Botha, E. *Chemosphere.* **1992**, *25*, 335-341.
14. Stolpe, N. B.; Shea, P. L. *Soil Sci.* **1995,** *160*, 359-370.
15. Flury, M. *J. Environ. Qual. 25*, 25-45.
16. Fink, R. J.; Flectchall, O. H. *Crop Sci.* **1962,** *19*, 81-83.
17. Fletchall, O. H. *The Effects on Subsequent Crops of Simazine Treatment of Corn; In* WSSA Abstracts 1958 Meeting; 1958, pp. 1-2.
18. Foy, C. L. *Weed control in Agronomic Crops: a Report of Progress;* Proc. California Weed Control Conf.; 1960, pp.66-72.
19. Frank, R.; Sirons, G. J.; Anderson, G. W. *Can. J. Soil Sci.* **1983,** *3*, 315-325.
20. Kells, J. J.; Leep, R. H.; Tesar, M. B.; Leavitt, R. A.; Cudnohufsky, J. *Weed Technol.* **1990,** *4*, 360-365.
21. Scudder, W. T. *Proc. SWC* **1959,** *12*, 187.
22. Sheets, T. J. *Residue Rev.* **1970,** *32*, 287-310.
23. Sheets, T. J.; Harris, C. I.. *Residue Rev.* **1965,** *11*, 119-140.
24. Best, J. A.; Weber, J. B. *Weed Sci.* **1974,** *22*, 364-373.
25. Hiltbold, A. E.; Buchanan, G. A.. *Weed Sci.* **1977**, *25*, 515-520.
26. McCormick, L.L.; Hiltbold, A.E. *Weeds* **1966,** *14*, 77-82.
27. Roeth, F. W.; Lavy, T. L.; Burnside, O. C. *Weed Sci.* **1968,** *17,* 202-205.
28. Sorenson, B. A.; Koskinen, W. C.; Buhler, D. D. ; Wyse, D. L.; Lueschen, W. E.; Jorgenson, M. D. *Int. J. Environ. Anal. Chem.* **1995,** 61, 1-10.

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch015

29. Burnside, O. C.; Wicks G. A.. *Weed Sci.* **1980,** *28*, 661-666.
30. LeBarron, H. M. *Residue Rev.* **1970,** *11,*119-140.
31. Lamoureux, G.L.; Simoneuax, B.; Larson, J. In *Triazine Herbicides Risk Assessment;* Ballantine, L.; McFarland, J.; Hackett, D., Eds.; *ACS Symposium Series 68;* American Chemical Society, Washington, DC, 1998, pp.60-81.
32. Frear, D.S.; Swanson; H.R. *Phytochemistry* **1970,** *9*, 2123-2132.
33. Esser, H.O.; Dupuis, G; Ebert, E; Vogel, C; Macro, G.J. In *s*-Triazines; Kearney, P.C.; Kaufman, D.D., Eds; *Herbicdes Chemistry, Degradation, and Mode of Action*; Marcel Dekker, Inc.: New York, N.Y. 1975 Vol 1, 2nd Edition; pp. 129-208.
34. Zablotowicz, R. M.; Hoagland, R. E ; Locke, M. A. In *Bioremediation Through Rhizosphere Technology; Anderson, T.A.; Coates, J. R.*, Eds.; ACS Symp. Ser. 563; ACS Books, Washington, DC, 1994, pp. 184-198..
35. Cook, A. M. *FEMS Microbiol. Rev.* **1987,** *46*, 93-116.
36. de Souza, M. L.; Newcombe, D.; Alvey, S.; Crowley, D. E.; Hay, A.; Sadowsky, M. J; Wackett, L. P. *Appl. Environ. Microbiol.* **1998**, *64*, 178-184.
37. de Souza, M. L.; Wackett, L. P.; Boundy-Mills, K. L.; Mandelbaum, R. T.; Sadowsky, M. J. *Appl. Environ. Microbiol.* **1995**, *61*, 3373-3378.
38. Erickson, E. L.; Lee, K. H. *Critical Rev. Environ. Cont.* **1989**, *19,* 1-13.
39. Mandelbaum, R.T.; Wackett, L.P.; Allan, D. L. *Appl. Environ. Microbiol.* **1993**, *59*, 1695-1701.
40. Mandelbaum, R. T., L. P. Wackett, and D. L. Allan. *Environ. Sci. Technol.* **1993**, *27*, 1943-1946.
41. Struthers, J. K.; Jayachandran, K.; Moorman, T. B. *Appl. Environ. Microbiol.* **1998**, *64*, 3368-3375.
42. Mulbry, W. W. *Appl. Environ. Microbiol.* **1994**, *60*, 613-618.
43. Nagy, I.; Compernolle, F.; Ghys, K.; Vanderleyden, J.; de Mot, R. *Appl. Environ. Microbiol.* **1995**. *61*, 2056-2060.
44. Yanze-Kontchou, C.; Gschwind, N. Appl. Environ. Microbiol. **1994,** *60*, 4297-4302.
45. Boundy-Mills, K. L.; de Souza, M. L.; Mandelbaum, R. T.; Wackett, L.P.; Sadowsky, M. J. *Appl. Environ. Microbiol.* **1997,** *63*, 916-923.
46. Bouquard, C.; Ouazzani, J.; Prome, J. C.; Michel-Briand, Y.; Plesiat, P. *Appl. Environ. Microbiol.* **1997,** *63*, 862-866.
47. Cook, A. M.; Hutter, R. *J. Agric. Food. Chem.* **1981,** *29*, 1135-1143.
48. LeBarron, H.; McFarland, J. E. In *Managing Resistance to Agrochemicals*; Green, M. B.; LeBarron, H. H.; Moberg, W. K. Eds.; ACS Symposium Ser. 421, ACS Books, Washington, D.C. 1990; pp. 336-352.
49. Behki, R.M.; Khan, S. U. *J. Agric. Food Chem.* **1986,** *34*, 746-749.
50. McMahon, P. B.; Chapelle, F. H.; Jagucki, M. L. *Environ. Sci. Technol.* **1992**, *26*, 1556-1559.
51. Radosevich, M.; Traina, S. J.; Hao, Y. L.; Tuovinen, O. H. *Appl. Environ. Microbiol.* **1995**, *61*, 297-302.
52. de Souza, M. L.; Sadowsky, M. J.; Wackett, L. P. J. Bacteriol. **1996**, *178*, 4894-4900.

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch015

53. Behki, R.; Topp, E.; Dick, E.; Germon, P. *Appl. Environ. Microbiol.* **1993**, *59*, 1955-1959.
54. Korpraditskul, R., A. Katayama, and S. Kuwatsuka. *J. Pest. Sci.* **1993**, *18*, 293-298.
55. Shao, Z.; Behki, R. *Appl. Environ. Microbiol.* **1995**, *61*, 2056-2060.
56. Shao, Z. Q.; Behki, R. *Appl. Environ. Microbiol.* **1995**, *61*, 2061-2065.
57. Eaton, R. W.; Karns, J. S. *J. Bacteriol.* **1991**, *173*, 1215-1222.
58. Eaton, R.W.; Karns, J. S. *J. Bacteriol.* **1991**, *173*, 1363-1366.
59. Karns, J. S.; Eaton, R. W. *J. Agric. Food Chem.* **1997,** *45*, 1017-1022.
60. Karns, J.S. *Appl. Environ. Microbiol.* **1999,** *65*, 3512-3517.
61. Nagy, I.; Schoofs, G.; Compernolle, F.; Proost, P.; Vanderleyden, J.; Demot, R. *J. Bacteriol.* **1995**, *177*, 676-687.
62. Nagy, I.; Verheijen, S.; De Schrijver, A.; Van Damme, J.;Proost, P.; Schoofs, G.; Vanderleyden, J.; De Mot, R. *Arch. Microbiol.* **1995,** *163*, 439-446.
63. Shao, Z. Q.; Sefens, W.; Mulbry, W.; Behki, R. M. *J. Bacteriol.* **1995**, *177*, 5748-5755.
64. Shao, Z. Q.; Behki, R. *Appl. Environ. Microbiol.* **1996**, *62*, 403-407.
65. Sadowsky, M. J.; Tong, Z.; de Souza, M.; Wackett, L. P. *J. Bacteriol.* **1997**, *189*, 152-158.
66. Martinez-Zayas, B; de Souza, M. J.; Wackett, L. P.; Sadowsky, M. J. In Abstracts from the 99th General Meeting of the American Society for Microbiology, Chicago, IL., 1999; Abstract Q-352, p. 601.
67. Seffernick, J. L.; Sadowsky, M. J.; Wackett, L. P. In Abstracts from the 99th General Meeting of the American Society for Microbiology, Chicago, IL., 1999; Abstract K-62, p. 412.
68. de Souza, M. L.; Wackett, L. P.; Sadowsky, M. J. *Appl. Environ. Microbiol.* **1998**, *64*, 2323-2326.
69. de Souza M. L.; Seffernick, J.; Martinez, B.; Sadowsky, M. J.; Wackett, L. P. *J. Bacteriol.* **1998**, *180*, 1951-1954.
70. Nakatsu, C.; Ng, J.; Singh, R.; Strais, N.; Wyndham, C. *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 8321-8316.
71. Nakatsu, C. H.; Wyndham, R. C. *Appl. Environ. Microbiol.* **1993,** *59*, 3625-3633.
72. Ng, J.; Wyndham, R. C. *Can. J. Microbiol.* **1993**, *39*, 92-100.
73. Wyndham, R. C.; Cashore, A.E.; Nakatsu , C. H.; Peel, M. C. **1994,** *5*, 323-342.
74. Topp, E.;Tessier, L.; Lewis, M. Abtsracts of the 97th General Meeting of the American Society for Microbiology, Miami Beach, FL, 1997; pp. 522.
75. Jutzi, K.; Cook, A. M.; Hutter, R. *Biochem. J.* **1982**, *208*, 679-684.
76. Karns, J. S.; Eaton, R. W. In Abstracts of the 91st General Meeting of the American Society for Microbiology, Dallas, TX, 1991; pp. 304.
77. Cook, A.M.; Beilstein, P.; Grossenbacher, H.; Hutter, R. *Biochem. J.* **1985**, *231*, 25-30.
78. Laskin, A. I.; Lechavalier, H. A. CRC Handbook of Microbiology. 2nd Ed. CRC Press, Bocca Raton, FL; 1984, pp. 557-567.
79. Ostrofsky, E. B.; Traina, S. J.; Tuovinen, O. H. *J. Environ. Qual.* **1997**, *26*, 647-657.
80. Vanderheyden, V.; Debongnie, P.; Pussemier, L. *Pest. Sci.* **1997**, *49*, 237-242.

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch015

81. Scholl, W.; Davis, R. O. E.; Brown, B. E.; Reid, F. R. *Chemtech.* **1973**, *29,* 202-205.
82. Hauck, R. D.; Stephenson, H. F. *J. Agric. Food Chem.* **1964**, *12,* 147-151.
83. Baker, B. D.; Richards, P. R. In *Rational Readings on Environmental Concerns;* Lehr, J. H., Ed.; Van-Nostrand Reinhold: New York, N.Y., 1992; pp. 84-97.
84. Cook, A. M.; Grossenbacher, H.; Hutter, R. *Experientia* **1983**, *39,* 1191-1198.
85. Cook, A. M.; Hutter, R. *J. Agric. Food Chem.* **1984**, *32,* 581-585.
86. Mandelbaum, R. T.; Wackett, L. P. In Abstracts of the 94th General Meeting of the American Society for Microbiology, Las Vegas, NV, Abstract Q-86, 1994; p.402.
87. Clennel, A. M.; Johnston, B.; Rawlings, D. E. *Appl. Environ. Microbiol.* **1995**, *61,* 4223-4229.
88. Coco, W. M.; Sangodkar, U. M. X.; Chakrabarty, A. M. *Adv. Appl. Biotechnol.* **1989**, *4,* 44-59.
89. Haugland, R. A.; Sangodkar, U. M. X.; Chakrabarty, A. M. *Mol. Gen. Genet.* **1990**, *220,* 222-228.
90. Lessie, T. G.; Wood, M. S.; Byrne, A.; Ferrante, A. *Pseudomonas: Biotransformations, Pathogenesis, and evolving biotechnology;* Silver, S.; Chakrabarty, A. M.; Iglewski, B.; Kaplan, S., Eds.; American Society for Microbiology, Washington, D.C., 1990; pp. 279-291.
91. Ruzzi, M.; Zennaro, E. *FEMS Microbiol. Lett.* **1989**, *59,* 337-344.
92. Sangodkar, U. M. X.;. Chapman, P. J.; Chakrabarty, A. M. *Gene* **1988**, *71,* 267-277.
93. Tomas, A. W.; Slater, J. H.; Weightman, A. J. *J. Bacteriol.* **1992**, *174,* 1932-1940.
94. Tomas, A. W.; Topping, A. W.; Slater, J. H.; Weightman, A. J. *J. Bacteriol.* **1992**, *174,* 1941-1947.
95. Tomasek, P. H.; Frantz, B.; Sangodkar, U. M. X.; Haugland, R. A.; Chakrabarty, A. M. *Gene* **1989**, *76,* 227-238.
96. Tsuda, M.; Iino, T. *Mol. Gen. Genet.* **1987**, *210,* 270-276.
97. Tsuda, M.; Iino, T. *Mol. Gen. Genet.* **1988**, *213,* 72-77.
98. Tsuda, M.; Minegishi, K.-I.; Iino, T. *J. Bacteriol.* **1989**, *171,* 1386-1393.
99. van der Ploeg, J.; Willemsen, M.; van Hall, G.; Janssen, D. B. *J. Bacteriol.* **1995**, *177,* 1348-1356.
100. Van Spanning, R. J. M.; Reijnders, W. M. N.; Stouthamer, A. H. *J. Bacteriol.* **1995**, *177,* 4772-4778.
101. Shelton, D. R, Karns, J. S.; McCarty, G. W.; Durham, D. R. *Appl. Environ. Microbiol.* **1997,** *63,* 2832-2835.

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch015

Downloaded by PENNSYLVANIA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch016

Chapter 16

Microbiol Reductive Dehalogenation in the Rhizosphere

Andrei L. Barkovskii

The Department of Civil and Environmental Engineering, 172 EWRE Building, The University of Michigan, 1351 Beal Avenue, Ann Arbor, MI 48109–2125

Accelerated microbial oxidative degradation of aryl and alkyl halides in the rhizosphere is a well-documented phenomenon. In contrast, microbial reductive dehalogenation in the rhizosphere remains relatively unexplored. Many agrochemicals undergo reductive dehalogenation transformation in soils and sediments. Reductive dehalogenation (RDE) is an advantageous process to microorganisms under anaerobic conditions. For bacterial RDE to occur, four main requirements must be present: (i) physico-chemical parameters (redox potential, pO_2, terminal electron acceptors, etc.) favorable to RDE reactions and dechlorinating microorganisms, (ii) bacteria capable of fortuitous or direct RDE, (iii) electron donors, along with organic and mineral nutrients; and, (iv) bioavailable halogenated compounds. In theory, the rhizosphere environment provides all the conditions required for bacterial RDE which is favored by certain rhizospheric processes. Since there is only limited evidence of bacterial RDE occuring in the rhizosphere, there is a need to ascertain the importance of this process in the degradation of anthropogenic organohalides. The mechanisms of microbial RDE and the suitability of rhizospheric conditions to the microbial RDE reactions are discussed in this chapter.

The ubiquity of synthetic halogenated aromatic, aliphatic, and heterocyclic compounds in the environment continues to be at the forefront of public and

In Pesticide Biotransformation in Plants and Microorganisms; Hall, J., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2000.

regulatory concern due to issues of bioaccumulation, recalcitrancy, and presumed estrogen mimetic properties of some of these compounds. These concerns have provided impetus for extensive evaluation of the fate of such chemicals in any given ecosystem (*1*). Nearly three decades of research on the fate of aryl and alkyl halides have shown a remarkable capability of soil microbiota to transform these compounds into non-toxic entities.

There are seven mechanisms of microbial dehalogenation (*2*). However, RDE is the only known biodegradation mechanism for certain significant pollutants including polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated biphenyls (PCBs), hexachlorobenzene (HCB), tetrachloroethene (PCE), heptachlor, aldrin, etc. (*3*). RDE mainly occurs under anaerobic conditions and is the initial step in anaerobic biodegradation of most aryl halides. Moreover, RDE is also involved in aerobic degradation of certain highly halogenated compounds. Hence, microbial RDE is of interest because of its involvement in the environmental fate of pesticides and industrial chemicals, and its potential application to bioremediation of pollutants and hazardous wastes.

Since the late 1970s, numerous reports have appeared in the literature demonstrating that plants may enhance the microbial degradation of several different halogenated compounds including: 2,4-dichlorophenoxyacetic acid (2,4-D) (*4*), dicamba (*5*), pentachlorophenol (PCP) (*6*), chlorobenzoates (*7*), trichloroethylene (TCE) (*8*), etc. Many studies describe the phenomenon, but rarely provide specific mechanisms of organohalide biodegradation under rhizospheric conditions. The most common explanation for the influence of plants on microbial metabolism of xenobiotics is increased microbial cell numbers and enhanced microbial activity in the rhizosphere due to carbon substrates provided by rhizodeposition. Other possible mechanisms include selection of highly effective degrading microorganisms; induction of appropriate enzymes with substrate analogs secreted by plants; enhanced water and solute transfer among the roots and the rhizosphere; etc. (*9*). While these hypothesis are plausible, the specific mechanisms of microbial degradation of halogenated compounds in the rhizosphere presently remain unclear. This is especially true with regard to the microbial RDE reactions in the rhizosphere. The physico-chemical parameters of the rhizosphere, the distribution of microorganisms capable of RDE in rhizospheric environments, and the bioavailability of organohalides in the rhizosphere remain to be assessed. The fundamentals of microbial RDE and the suitability of rhizospheric environments for microbial RDE reactions are discussed in this chapter.

Dehalogenation Mechanisms

The carbon-halogen bond can be cleaved by either enzymatic dehalogenation (dehalogenases), or by spontaneous chemical dehalogenation. However, various scenarios occur, ranging from fortuitous dehalogenation by an enzyme with broad substrate specificity to a substarte-specific dehalogenase. Enzymes with broad substrate specificity catalyze the conversion of halogenated analogs of the

Downloaded by PENNSYLVANIA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch016

Downloaded by PENNSYLVANIA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch016

corresponding unsubstitued substrates or of related compounds. This might lead to "fortuitous" dehalogenation of the substrate analog. Spontaneous dehalogenation reactions may occur as a result of chemical decomposition of unstable primary products. Thus, the distinction of whether such a reaction is direct or "fortuitous" may be hard to ascertain.

Seven mechanisms of microbial dehalogenation are known (Table I) (*2*): (i) RDE where the halogen substituent is replaced by hydrogen; (ii) oxygenolytic dehalogenation catalyzed by mono- or dioxygenases which incorporate one or two atoms of molecular oxygen into the substrate; (iii) hydrolytic dehalogenation catalyzed by halidohydrolases where the halogen substituent is replaced in a nucleophilic substitution reaction by a hydroxyl group derived from water; (iv) "thiolytic" dehalogenation by a specific glutathione S-transferase (e.g., in dichloromethane-utilizing bacteria) catalyzes the formation of a S-chloromethyl glutathione conjugate, with concomitant dechlorination; (v) intramolecular substitution where nucleophilic displacement yielding epoxides with concomitant dehalogenation; (vi) dehydrohalogenation, where HCl is eliminated from the molecule, leading to double bond formation, and; (vii) hydration, a hydratase-catalyzed addition of a water molecule to an unsaturated bond leading to dehalogenation of vinylic compounds, such as 3-chloroacrylic acid, *via* chemical decomposition of an unstable intermediate.

Since the enzymes responsible for dehalogenation reactions can be induced either by natural substrate analogs or structurally unrelated compounds, it is unclear whether they are genuinely adaptive to anthropogenic halogenated compounds or are part of evolutionary old natural catabolic pathways (*10*). One logical theory is that the presence of the xenobiotics merely increases the natural biodegradative activity, which is normally induced by natural substrates (*11*). A natural input of halogenated chemicals into the environment originates from plants, microorganisms, marine organisms, and natural processes such as forest fires (*12*). Haloalkanes constitute most of the 2000 naturally occurring organohalogen compounds identified to date; however, chlorinated pyrroles and indoles, as well as phenols, phenolic ethers, and benzenes are also produced naturally (*13*). The marine environment is a rich source of halogenated compounds such as chlorinated and brominated terpenes, amino acids and pyrroles (*14*), and aliphatic and aromatic compounds (*13*). The structural features of naturally occurring aryl halides (Figure 1) indicate similarities with so-called truly anthropogenic aryl halides.

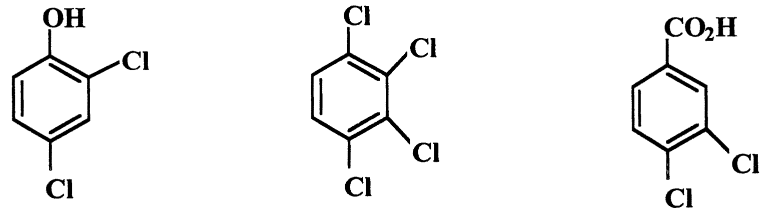
The ubiquity of halogenated compounds in the environment facilitates natural selection and the evolution of catabolic pathways. Several theories have been invoked to explain the adaptability of microbial communities and populations to novel environmental niches, including intra- or interspecific genetic exchange, gene rearrangements and mutations (*10, 15*). Microbial versatility is thus considered a reflection of the adaptation mechanisms that have taken place to confer novel catabolic abilities into the microbial catabolic gene pool.

Downloaded by PENNSYLVANIA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch016

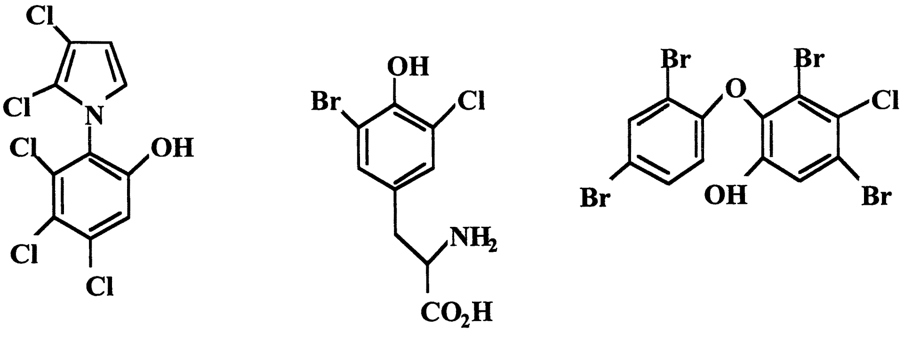
Table I. Types of Dehalogenation Mechanisms

Type of Dehalogenation	*Generic Examples of Dehalogenation Reaction*
Reductive Dehalogenation	$R\text{-}Cl + 2H^+ + 2\,e^- \rightarrow R\text{-}H + HCl$
Oxygenolytic Dehalogenation	$COOH\text{-}R\text{-}Cl + O_2 + NADH + H^+ \rightarrow R\text{-}(OH)_2 + NAD^+ + CO_2 + HCl$
Hydrolytic Dehalogenation	$R\text{-}CH_2Cl + H_2O \rightarrow R\text{-}CH_2OH + HCl$
"Thiolytic" Dehalogenation	$CH_2Cl_2 + GSH \rightarrow [GS\text{-}CH_2Cl_2] + HCl$ $[GS\text{-}CH_2Cl] + H_2O \rightarrow GS\text{-}CH_2OH + HCl$ $GS\text{-}CH_2OH \rightarrow CH_2O + GSH$
Intramolecular Substitution	$R\text{-}CHOH\text{-}CH_2Cl \rightarrow R\text{-}CH(O)CH_2 + HCl$
Dehydrohalogenation	$RCl_6 \leftrightarrow RCl_5 + HCl$
Hydration	$HOOC\text{-}CH{=}CHCl + H_2O \rightarrow [HOOC\text{-}CH_2\text{-}CHOHCl]$ $[HOOC\text{-}CH_2\text{-}CHOHCl] \rightarrow HOOC\text{-}CH_2\text{-}CHO + HCl$

Downloaded by PENNSYLVANIA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch016


OH
Cl
Cl
Cl
Cl
Cl
Cl
CO_2H
Cl
Cl

2,4-dichlorophenol **1,2,3,4-tetrachlorobenzene** **3,4-dichlorobenzoate**


Cl
Cl
N
Cl
OH
Cl
Cl
OH
Br
Cl
NH_2
CO_2H
Br
Br
O
Cl
Br
OH
Br

neopyrrolomycin **3-bromo-5-chlorotyrosine** **2,4,2',4'-tetrabromo-3-chloro-6-hydroxy-diphenylether**

Figure 1. Molecular structures of some naturally-occurring halogenated aromatic compounds.

Reductive Dehalogenation

Reductive dehalogenation (RDE) is a two-electron transfer reaction involving release of the halogen as a halogenide ion and its replacement by hydrogen (Table I). Even though RDE activity has been reported for the aerobic degradation of organohalides such as PCP (*16*), dichlorobenzoate (*17*), and some others, RDE primarily occurs in anaerobic systems. In fact, for highly chlorinated biphenyls, hexachlorobenzene, and some other highly chlorinated compounds, anaerobic RDE is the only known biodegradation mechanism (*3*). Even the most recalcitrant compounds such as PCDDs, recently were shown to undergo microbial reductive dechlorination in anaerobic soils (*18*) and sediments (*19, 20*). In addition, RDE reactions are involved in transformation of many highly halogenated pesticides (Table II) in the soil environment.

Like other anaerobic processes, RDE typically occurs in complex, mutualistic microbial communities. Despite the apparent ubiquity of this process in anoxic habitats, knowledge of the microorganisms and the physiology of RDE is limited, predominantly due to extreme difficulties in isolating microorganisms capable of catalyzing these reactions (*21, 22*). There are only a few examples of pure cultures which can accomplish RDE reactions; e.g., *Desulfomonile tiedje* DCB-1 that dechlorinates chlorobenzoates and chlorophenols (*3*), and strain NTB-1 (formerly *Alcaligenes denitrificans* NTB-1) that dechlorinates 2,4-D (*17*).

Concerning the mechanism of RDE, a reduced organic substrate or H_2 can be the source of both the reducing power and the protons (one-step transfer of two electrons and one proton. Dehalogenation might also occur in a two-step reduction by an electron donor (reduced organic substrate) and with proton abstraction from a solvent. There are two principal mechanisms of RDE. The first and the most common is microbial cometabolic RDE which can be fortuitous or not. Cometabolic transformation reactions do not yield energy for microorganisms, or will not result in cell growth. Therefore microorganisms performing cometabolic RDE use not organohalides, but other compounds as terminal electron acceptors. The second is RDE *via* halorespiration where organohalides are used as terminal acceptors of electrons by microorganisms able to yield energy from this electron transfer.

In cometabolic transformation, RDE is catalyzed either by enzymes with broad specificity or by transition metal cofactors. One example is the ammonia monooxygenase in *Nitrosomonas europea,* which reduces the trichloromethyl group of nitrapyrin, rather than oxygen (*23*). Transition metal cofactors such as vitamin B_{12} (cobalamin) are produced by many anaerobic, in particular fermentative and methanogenic organisms, that participate in cometabolic transformation. Different classes of halogenated compounds such as alkyl and aryl halides, and heterocyclic compounds, were shown to undergo RDE *via* transition metal cofactor catalysis (*2, 3, 24*). Another subcategory of cometabolic RDE processes pertinent to rhizospheric environments is dehalogenation through microbial respiration of humic acids, their constituents and other ubiquitous polyphenolic molecules. In this case, quinone and semiquinone moieties of humic materials act as terminal electron acceptors for microorganisms. Reduced humic compounds, in turn, transfer these excessive

Downloaded by PENNSYLVANIA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch016

Downloaded by PENNSYLVANIA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch016

Table II. Common Agrochemical Organohalides that Undergo Reductive Dehalogenation Transformation in Soil

Common Name	*Chemical Name*
Aldrin	1,2,3,4,10,10-Hexachloro-1,4-endo,exo-5,8-dimethanonaphthalene
Benthiocarb	*S*-4-Chlorobenzyl-*N*,*N*-diethyl thiocarbamate
Bromacil	5-Bromo-3-*sec*-butyl-6-methyl uracil
Bromoxynil	3,5-Dibromo-4-hydroxybenzonitrile
Chloronitrofen	4-Nitrophenyl-2,4,6-trichlorophenyl ether
DDT	1,1,1- Trichloro-2,2-bis(*p*-chlorophenyl)ethane
Dicamba	3,6-Dichloro-2-methoxybenzoate
Heptachlor	1,4,5,6,7,8,8-Heptachloro-3a,5,7,7,7a-tetrahydro-4,7-methanoindene
Lindane	√-1,2,3,4,5,6-Hexachlorocyclohexane
Methoxychlor	1,1,1-Trichloro-2,2-bis(*p*-methoxyphenyl)ethane
Mirex	Dodecachlorooctahydro-1,3,4-metheno-2*H*-cyclobuta(c,d)pentalene
PCP	Pentachlorophenol
Picloram	4-Amino-2-carboxy-3,5,6-trichloropyridine
Techloftham	*N*-(2,3-Dichlorophenyl)3,4,5,6-chloro-phthalamic acid
TPN	2,4,5,6-Tetrachloroisophthalonitrile
Toxaphene	Mixture of isomeric hepta-/octa-/nonanchlorobornanes

Downloaded by PENNSYLVANIA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch016

electrons to highly oxidized compounds present in the system (*25-27*). Such interactions cause the redistribution of electrons in the system, acceleration of dehalogenation of organohalides, and alteration of their dehalogenation patterns (*28*).

Intracellular mechanisms of cometabolic RDE are presently poorly understood. For methanogenic bacteria, Fathepure and Boyd (*29*) presented a hypothetical scheme linking RDE of tetrachlorethene to methanogenesis. This scheme suggested that some electrons generated during methanogenesis may have been nonspecifically transferred to tetrachlorethene by an electron carrier whose main function is to deliver electrons for the methane generating system. Furthermore, it was suggested that this carrier could be the Ni containing factor F_{430}, a prosthetic group of methyl-coenzyme *M* reductase that mediates the final step in methanogenesis. Later studies (*30*) have clearly demonstrated the above contention using coenzyme F_{430} isolated from *Methanobacterium thermoauthotroficum.* Similar evidence exists on the involvement of F_{430} and methyl-coenzyme *M* in the RDE of dichloroethane (*31*). Many microorganisms also contain high levels of vitamin B_{12} and corrinoids with dechlorinating activity on PCBs (*32*) , PCDDs (*24*) and other halogenated compounds. Other transition metal-containing and redox carrier molecules such as Fe-porphyrins and cytochromes may also transfer electrons to halogenated compounds in anaerobic bacteria. The relationship between the direct energetic metabolism and cometabolic transformation of halogenated compounds is schematically presented in Figure 2.

All the examples of RDE reactions described above are unable to support microbial growth because no energy is directly gained by microorganisms from such a transfer of electrons. However, thermodynamic considerations indicate that RDE is usually exergonic and expected to yield free energy. Hexachloroethane can be a stronger electron acceptor than oxygen. Therefore, energy calculations (*33*) indicate that the reduction of hexachloroethane to pentachloroethane is favorable, even under aerobic environments, and yields a large amount of free energy. Similarly, tetrachloromethane has a higher reduction potential than the NO_3^-/NO_2^- redox pair, and therefore can be a better electron acceptor than NO_3^-. Interestingly, the redox potential of many other chlorinated compounds (*33*) has been found similar to that of the NO_3^-/NO_2^- redox couple (E'_0 = 433 mV). Also, these values are much higher than redox-potentials for sulfidogenic (-220 mV) and methanogenic (-240 mV) reactions. Theoretical calculations suggest that RDE of halogenated compounds would yield in excess of up to five times more free energy per mole of reduced electron acceptor than can be produced in sulfidogenic or methanogenic reactions. Therefore, a metabolic role for chlorinated compounds as terminal electron acceptors to anaerobic microorganisms is conceivable. This consideration has led to the search for microorganisms that use halogenated compounds as energy-yielding electron acceptors. The capacity of halogenated compounds to serve as terminal acceptors in anaerobic environments is now well-known (*3, 34*). A wide variety of both aliphatic and aromatic compounds can support halo-respiration under anoxic conditions (*33*). Some of the microorganisms capable of halo-respiration are presented in Table III (33). The coupling of dehalogenation to energy production has great environmental

Downloaded by PENNSYLVANIA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch016

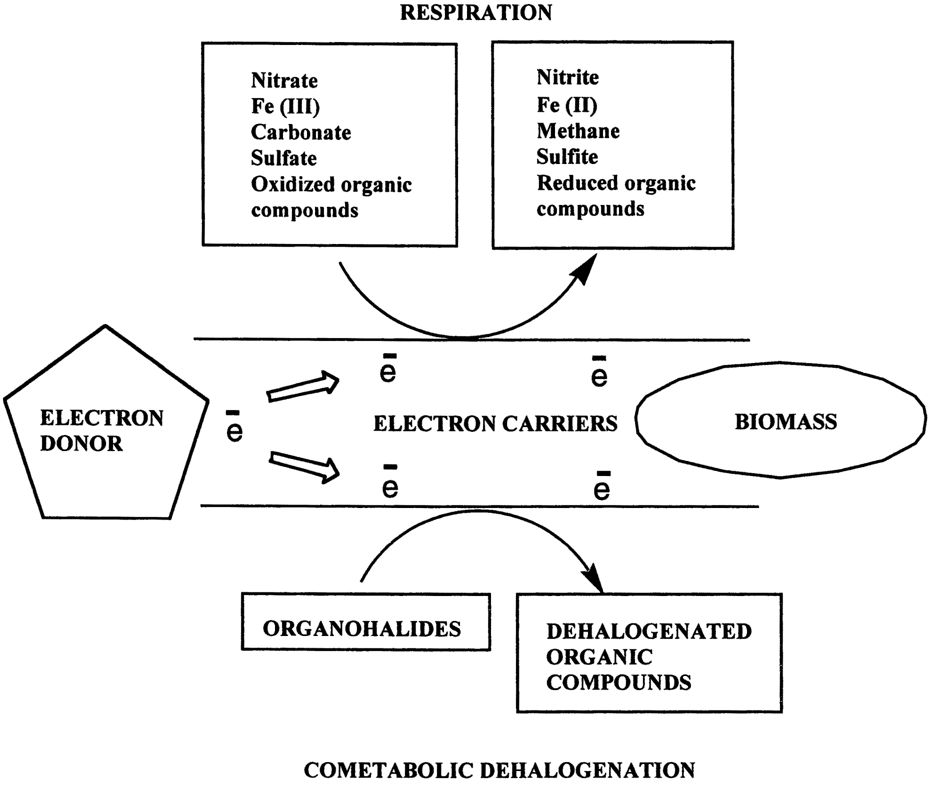

RESPIRATION
Nitrate
Fe (III)
Carbonate
Sulfate
Oxidized organic compounds
Nitrite
Fe (II)
Methane
Sulfite
Reduced organic compounds
ELECTRON DONOR
e
e
e
e
e
ELECTRON CARRIERS
BIOMASS
ORGANOHALIDES
DEHALOGENATED ORGANIC COMPOUNDS
COMETABOLIC DEHALOGENATION

Figure 2. The relationship between direct energetic metabolism and cometabolic dehalogenation in microorganisms.

Downloaded by PENNSYLVANIA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch016

Table III. Pure Bacterial Cultures with the Capability to Respire Organohalides

Culture	*Electron Donor*	*Electron Acceptor*
Desulfobacterium sp. PCE1	L-Lactate, Formate, Butyrate, Pyruvate, Ethanol	Perchloroethene, Pentachlorophenol, Sulfite, Thiosulfate
Dehalobacter restrictus PER-K23	H_2	Perchloroethene
Dehalospirillum multivorans	H_2, Pyruvate, Formate, Lactate, Ethanol, Glycerol	Perchloroethene, Fumarate, NO_3^-
Dehalococcoides ethenogenes 195	H_2	Perchloroethene, Trichloroethene, 1,2-Dichloroethane
Desulfomonile tiedjei DCB-1	Pyruvate, Acetate, H_2-CO_2, Formate, Benzoate, 3-Chlorobenzoate	3-Chlorobenzoate, Sulfate, Sulfite, Thiosulfate
Desulfitobacterium hafniense DCB-2	Pyruvate, Tryptophan	3-Chloro-4-hydroxyphenylacetate, Sulfite, Thiosulfate, Nitrate, Fe (III)
D. dehalogenans JW/IU-DC-1	Pyruvate, Lactate, Formate, H_2	3-Chloro-4-hydroxyphenylacetate, 2,4-Dichlorophenol, Sulfite, Thiosulfate, Nitrate, Fumarate
D. chlororespirans Co23	Pyruvate, Lactate, Formate, H_2, Sulfur, Sulfite	3-Chloro-4-hydroxyphenylacetate, 3-Chloro-4-hydroxybenzoate, 2,3- and 2,6-Dichlorophenols, Thiosulfate
D. frappieri PCP-1	Pyruvate	Pentachlorophenol, Sulfite, Thiosulfate, Nitrate

significance, since such reactions selectively enrich halo-respiring populations in various environments, including the rhizosphere.

The understanding of the mechanism of energy conservation through halo-respiration is presently developing. To generate ATP *via* RDE, bacteria employ specific enzymes and cofactors that recognize chlorinated compounds as terminal electron acceptors. A direct proof of bacteria using chlorinated hydrocarbons as terminal electron acceptors came from Griffin *et al.* (*35*). In this experiment, *D. tiedjei* cells were incubated with 2,5-dichlorobenzoate in D_2O. Deuterium was exclusively incorporated at the site of dechlorination on the molecule. These results indicated that the proton came from the solvent while the electrons were derived from the electron donor, thus documenting that 3-chlorobenzoate served as a terminal electron acceptor. Mohn and Tiedje (*36*) presented evidence for chemiosmotic coupling of RDE and ATP synthesis in *D. tiedjei.* In this scheme, 3-chlorobenzoate served as the final electron acceptor, and energy was generated while electrons were being actively transferred *via* the electron-transport chain from an electron donor to 3-chlorobenzoate *via* a proton gradient across the membrane.

In contrast to cometabolic RDE, specific enzymes are involved in halo-respiration. Some enzymes have been recently purified and characterized, for instance, a membrane-associated 3-chlorobenzoate-dechlorinase from *D. tiedjei* (*37*). In another study (*38*), an aryl dehalogenase from the halo-respiring organism, *D. chlororespirans* Co23, has been partially characterized and found also to be membrane-associated. Neither NADPH nor NADH (redox = – 320 mV) supported reductive dechlorination, however reduced methyl viologen (redox = – 440 mV) was an electron donor for both membrane-bound dehalogenases. A few specific reductive dehalogenases such as tetrachloro-*p*-hydroquinone reductive dehalogenase from a *Flavobacterium* sp. (*39*) and a dehalogenase from *Dictyostelium discouideum* (*40*) use glutathione as an electron donor.

Physico-Chemical Parameters Favorable to Reductive Dehalogenation and Their Occurrence in the Rhizosphere

Microorganisms capable of respiratory or cometabolic RDE transformation processes are common in soil and sediment environments. Nevertheless, information on microbial RDE in rhizospheric environments is scarce. Whether the rhizosphere is a suitable environment for RDE reactions remains unclear. Conditions favorable for microbial RDE and their occurrence in the rhizosphere are considered below.

The most important physico-chemical factors for microbial RDE are oxygen content, corresponding redox potential, and availability of electron acceptors suitable for cometabolic transformation of halogenated compounds. It is widely assumed, although not always true, that oxygen inhibits RDE. This assumption is based on the observation that RDE typically occurs in natural environments only when they are anaerobic with low redox potential values (*41, 42*). Exceptions to inhibition by oxygen do exist, however, including reduction of hexachloroethane to PCE (apparent vicinal reduction) under presumably aerobic conditions (*43*), aryl hydrogenolysis of

Downloaded by PENNSYLVANIA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch016

PCP by pure cultures of *Flavobacterium* sp (*16*) and *Rhodococcus* sp (*44*), and 2,4-dichlorobenzoate dehalogenation by *Alcaligenes denitrificans* (*17*). Despite these exceptions, it is generally assumed that microbial RDE requires anaerobic conditions.

Oxygen concentration and corresponding redox potential in the rhizosphere vary among plants and environments, and even within individual rhizospheres. Some plants possess active mechanisms for delivering oxygen into the rhizosphere. The pathways of oxygen transport in plants through the roots and into the soil are not well understood. However, plants seem to fall into three general categories with respect to their varying oxygen-ventilating efficiencies: (i) non-wetland herbaceous and woody plants with poor ventilating capabilities; (ii) wetland woody plants with moderate ventilating capacities; and, (iii) wetland herbaceous plants with generally good ventilating capacities (*45*). Oxygen is supplied to the roots through soil diffusion as well as through plant transport. In many wetland plants, upon flooding, several physiological changes occur and more oxygen is transferred via the plant. Those plants are able to absorb oxygen through their leaves, lentices of twigs, stems, bark, and even the unflooded roots. Disintegration of the xylem cells (in response to ethylene) results in the formation of aerenchyma, i.e., long extended passages for the diffusion of atmospheric oxygen to the roots. From the roots, oxygen diffuses into the rhizosphere. Thus, submerged plants with this response may generate a rhizosphere that is more aerated (and have higher redox potential) than the surrounding soil/sediment. On the other hand, terrestrial plants are presumed to have lowered oxygen contents and redox potential in the rhizosphere due to the root and microbial respiration.

Anaerobic and aerobic compartments typically coexist in the rhizosphere of wetland plants. For wetland plants under flooding conditions, the highest oxygen concentration (and redox potential) is encountered at the root tips. Oxygen concentration and redox potential sharply decrease with increasing distance from the root tips and roots. For example, the radius of the oxidized microzone in the rhizosphere of flooded rice varies from less than 1 to 4 mm (*46*). Similarly, the radial extension of the oxidized zone (higher redox potential than the bulk soil) around the root tips of submerged *Myriophyllum verticillatum* L. and *Ranunculus circinatus* L. plants do not extend further than 1 mm (*47*). There are also diurnal cycles of oxygen enrichment in rhizospheric environments. The concentration of oxygen in the oxidized microzone of *Cymodocea rotundata* under daylight conditions was up to 75% of air saturation at the root surface, and this microzone was 80 μm thick (*48*). Under dark conditions, the concentration of oxygen at the root surface decreased to 20% of air saturation, and the thickness of the oxidized microzone shrank to 50 μm. The oxidized microzone around roots comprised about 0.05% of the total volume of the seagrass rhizosphere, and the root-mediated oxygen supply was estimated to be insignificant for the whole sediment oxygen budget in the rhizosphere. Therefore, the release of oxygen through plant transfer into the rhizosphere protects wetland plants under flooding from chemically hostile environmental conditions, but does not significantly impact the oxygen and redox state of the entire rhizosphere. Muramatsu et al. (*49*) reported a drastic decrease of redox potential values far below -100 mV in a "bulk" *Oryza sativa* (rice) rhizosphere after flooding. In unsaturated soils, some

Downloaded by PENNSYLVANIA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch016

wetland plants grow under anaerobic rhizosphere conditions and have higher levels of anaerobic by-products than non-wetland plants (*45*).

The rhizosphere of terrestrial plants, on the other hand, can be more reduced and less aerobic than bulk soil. In contrast to wetland plants, the root tips and surrounding soil often have the lowest oxygen levels in the rhizosphere of terrestrial plants. This effect has been observed in the rhizosphere of pea (*Pisum sativum*), vetch (*Vicia sativa*), sunflower (*Helianthus annuus*), but not in wheat (*Triticum aestivum*) and oat (*Avena sativa*) (*50*). Microelectrode and modeling studies of oxygen distribution in roots of corn (*Zea mays*) indicate that, in reduced soils, even the bulk of the root cap proper is likely to be anoxic, and this anoxia may be the norm for the plant. (*51*). Therefore, due to spatial and temporal heterogeneity in oxygen distribution, the rhizosphere environment usually, although not always, provides microorganisms with localized anaerobic and low redox potential conditions favorable to RDE reactions.

Except for halo-respiration, where halogenated compounds play the role of terminal acceptors of electrons, electron acceptors other than organohalides are required in rhizospheric environments for dechlorinating microorganisms. Fortunately, most traditional terminal electron acceptors are compatible with cometabolic RDE reactions. Carbon dioxide (*52, 53*), ferric (III) iron and nitrate (*54, 55*); organic acids (*52,* 56) and sulfate (*56*) were found to support pure cultures of microorganisms that catalyze cometabolic dehalogenation reactions. Traditionally, sulfate has been widely considered as an inhibitor of RDE. Whereas some studies report this inhibition (*3*), an increasing number of recent studies show that sulfate reducing conditions are suitable for RDE (*56, 57*). Most of the terminal electron acceptors, such as nitrate (*58*), ferric iron (*59, 60*), sulfate (*61*), and carbon dioxide (*59, 62*) are abundant in the rhizosphere, particularly in the rhizosphere of flooded plants, marsh- and sediment-dwelling plants, paddy soils, and wetlands (*63*).

Recently it was demonstrated that quinone moieties act as terminal electron acceptors for microorganisms (*25, 26*). In the rhizosphere, hydroxylated aromatic compounds, such as humic and fulvic acids are the main sources of quinone moieties. A variety of quinones and similar compounds are delivered into the rhizosphere with root exudates (*64*) and produced by microorganisms (*65, 66*). Total quionone and phenolic content in the rhizosphere might be as high as 0.1 mM (*67, 68*). Humic compounds are abundant in the rhizosphere due to microbial decomposition of dead biomass (plants and, to a lesser extent, other organisms) and further microbial diagenesis of soil organic matter (69, 70). Microbial reduction of quinone moieties enhances the capability of microorganisms to reduce other contaminants in the environment because microbially reduced quinone moieties shuttle electrons to other highly oxidized compounds (*25, 27*). In this manner, the presence of quinone moieties in the environment enhances the rates and the extent of microbial RDE reactions (*28*). For instance, the presence of 3,4-dihydroxybenzoic acid resulted in a 1.5 to 6-fold increase of the rates of bacterial reductive dechlorination of PCDDs congeners in anaerobic microcosms (*20*). In principle, humic and fulvic acids are able to shuttle electrons from a suitable electron donor (not necessarily microorganisms) directly to highly halogenated compounds such as PCDDs (*71*).

Downloaded by PENNSYLVANIA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch016

Thus, humic substances might be expected to be involved in RDE reactions in rhizospheric environments.

Bacteria Capable of Fortuitous or Direct Reductive Dehalogenation

RDE is not an abnormal event in the microbial world. This type of reaction is not intrinsically different from other microbially catalyzed redox reactions. The ecological aspects of the process are subject to the same rules as other microbial degradation processes. In the case of halo-respiration, the logic of RDE for microorganisms that perform this process is clear. These microorganisms are able to harness the energy derived from RDE. Some profit for microorganisms can be gained even from so-called "fortuitous" RDE through scavenging of 'hot' electrons to protect cell structures from 'overheating'. RDE is advantageous to microorganisms under anaerobic conditions because it provides an electron sink that allows reoxidation of metabolic intermediates. It is possible that the issue of detoxification of halogenated compounds is involved in the process of microbial "fortuitous" RDE as well. Therefore, "fortuitous" dehalogenation may not necessarily be truly incidental and may involve selection of specific microorganisms. Organohalides in rhizospheric environments could be a selective factor for microorganisms capable of RDE, as long as there are appropriate physico-chemical conditions for RDE and microbial communities suitable for this selection.

Such microbial communities are abundant in the rhizosphere. Most of the terminal electron acceptor processes (TEAP) compatible with RDE occur in the rhizosphere. There are many reports concerning sulfate and iron reducing microbial activity in the rhizosphere, mainly of wetland plants (*59, 61, 72,73*). Methanogens are also well-represented in the rhizosphere of wetland plants (*59, 74, 75*) where they typically co-exist with iron and sulfate reducing microorganisms (*59*, *75*). Sulfate reduction was found quite prominent in the rhizosphere of marine macrophytes (*61*) and salt marsh plants where populations of sulfate reducing bacteria reached $>10^7$ cells per ml of salt marsh sediments (*72*). These populations were primarily associated with root surfaces. In terrestrial agricultural environments, sulfate reducing activity was associated with anaerobic transformation of the acetanilide herbicides alachlor, propachlor and metolachlor (*73*).

Another group of microorganisms potentially capable of RDE and quite common in the rhizosphere are denitrifiers. Here, both terrestrial and wetland plants teem with these microorganisms. For instance, population density of culturable nitrate dissimilatory *Pseudomonas* spp. was reported to be twice as high in the rhizosphere of *Linum usitatissimum* (flax) and *Lycopersicon esculentum* (tomato) plants as that in bulk soil (*76*). The proportion of true denitrifiers among them gradually and significantly increases upon approaching the root vicinity. In fact, the rhizosphere was reported as the major site for coupled nitrification-denitrification activity in planted rice soils (*77*).

Downloaded by PENNSYLVANIA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch016

Microorganisms capable of humic constituent respiration are present in the rhizosphere as well. For instance, a laccase-positive strain of *Azospirillum lipoferum* 4T isolated from rice rhizosphere was reported to respire 3,4-dihydroxybenzoic and 3,4-dihydroxycinnamic (caffeic) acids (*25, 26*). A recent study suggests that such atypical non-motile laccase-positive strains like 4T originate from regular motile, laccase-negative strains in rice rhizosphere under extremely low oxygen concentrations *via* a two-step phenotypic switching event (*78*). Inoculation of rice roots with a regular motile, laccase-negative strain showed that rice rhizosphere enhanced the frequency of appearance of stable non-motile forms. This resulted in a 10-fold increase of non-motile forms in *A. lipoferum* population, compared to the population in inoculated plant growth medium (*79*). Interestingly, rice roots were found to exude alkylresorcinols, in particular 5-(12-heptadecenyl)-resorcinol (*80*), that likely could serve as terminal acceptors of electrons for those polyphenol-respiring microorganisms.

There is little or no information on the presence of halo-respiring microorganisms in the rhizosphere. Pure cultures of halo-respiring microorganisms have been isolated from anaerobic sewage (*81*), methanogenic consortia of microorganisms (*82*), sediments (*83*) and even rat intestine (*84*). All of these grew on simple carbon substrates and used a wide range of other electron acceptors in addition to halogenated compounds. Rhizospheric conditions do not preclude halo-respiration reactions and the existence of halo-respiring. There is indirect evidence that *Azospirillum* spp., could be polyphenol-respiring, and halo-respiring microorganisms. Patnaik *et al.* (*85*) observed a significant increase in the population of *Azospirillum* spp. in rice rhizospheres following application of 2,4-D (5 to 10 ppm) under flooded conditions, while the populations of *Azotobacter* and anaerobic nitrogen fixers were inhibited. The root and rhizosphere associated nitrogenase activity were significantly elevated after amendment with 2,4-D. *A. lipoferum* 4T nitrogenase activity is enhanced by respiration of humic constituents (*25, 26*). The appearance of polyphenol- and 2,4-D-respiring *Azospirillum* spp. could have originated *via* a two-step phenotypic switching event, under low oxygen conditions (*78, 79*).

Electron Donors and Nutrients in the Rhizosphere

The effects of electron donors and other nutrients on RDE have been examined even less extensively than the effects of TEAP. These nutrients include carbon, nitrogen, phosphate sources, and micronutrients. Sugars, organic acids, amino acids, methanol, formate, and H_2 have been found to stimulate dehalogenation (*3*). Undefined substrates such as yeast extract, trypticase, rumen fluid, sludge supernatant, and alfalfa (*Medicago sativa*) also stimulate dehalogenation. Finally, natural and slowly degraded materials such as humic matter also support dehalogenation (*3*). Many of these materials serve both as electron donors for RDE and as carbon sources for dehalogenating microorganisms. Additionally, if dehalogenation activity requires a population succession (e.g., to establish a low redox potential) or syntrophy (e.g., to provide vitamins or to prevent inhibitory product accumulation), nutrients might

Downloaded by PENNSYLVANIA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch016

stimulate dehalogenation by supporting the growth of non-dehalogenating microorganisms (*3*).

The rhizosphere contains elevated concentrations of carbon substrates compared to bulk soil. In fact, most of the electron donors and growth supporters mentioned above are more abundant in the rhizosphere than in bulk soil. The general increase in microbial cell numbers and microbial activity is presently the most common explanation for enhanced biodegradation potential in the rhizosphere. Nutrient fluxes in the rhizosphere have been thoroughly discussed elsewhere; e.g., Lynch and Whipps (*86*). Rhizo-deposits were found to support the growth of bacteria able to degrade aryl halides such as PCP, and the microorganisms retained their ability to metabolize the contaminant (*87*).

Other nutrients are also more readily available in rhizospheric environments due to both rhizodeposition and the mobilization of soil nitrogen, phosphorus, and other biogenic elements. The latter may occur through extraction by root exudates and evapotranspiration. For instance, ***Betula papyrifera*** Marsh (paper birch) root exudates were found to increase soil available carbon and microbial acquisition of soil nutrients (*88*). In forest ecosystems, the rhizosphere accumulates more acidity, base cations, nitrogen, and phosphorus than the bulk soil (*89*). Hydrogen may be present in the rhizosphere, which is considered to be an important electron donor for RDE, particularly under methanogenic conditions (*3, 20*). Hydrogen in the rhizosphere originates either from nitrogen fixation (*90, 91*) or from microbial fermentative metabolism of organic acids. Finally, plants may release compounds such as l-carvone and other terpenes that induce biodegradation of halogenated compounds, in particular PCBs (*92, 93*).

Bioavailability of Contaminants in the Rhizosphere

The fate of hydrophobic contaminants in the environment is controlled by both physico-chemical factors (sorption, photolysis, volatilization, etc.) and biological factors (biodegradation, bioaccumulation, etc.). The bioavailability of hydrophobic contaminants (defined here as the accessibility of natural organic matter-associated contaminants for microbial attack) generally determines their consequent transformation and mineralization (*94, 95*). Polyhalogenated aryl halides and structurally related compounds fall under the same bioavailability limitations as other hydrophobic contaminants (*1, 96*). With respect to more hydrophilic compounds, such as alkyl halides, bioavailability usually is not a limiting factor for biotransformation.

Soil architecture controlls microbially-mediated decomposition processes in terrestrial ecosystems (*97*). Soil organic matter (SOM) can be physically protected in soil, and thus unavailable to microorganisms. Mechanisms to explain this include: adsorption of organics on inorganic clay surfaces, and entrapment of materials in aggregates or in places inaccessible to microbes. Hydrophobic contaminants in soils and sediments are likewise protected by soil architecture. Non-extractable residues of

Downloaded by PENNSYLVANIA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch016

organic xenobiotics in soil can considerably exceed the amount of extractable residues (*98*).

Relatively few studies have examined the effect of the rhizosphere on the bioavailability of hydrophobic contaminants sorbed in the SOM. Plants can potentially affect the fate of sorbed hydrophobic organohalides by decreasing or increasing mass transfer from SOM to the degrading bacteria. Solar-driven transport processes (*99*) and extraction with root exudates (*100*) due to the presence of biosurfactants, organic ligands (*101*) and simple organic acids (*100*) may increase the aqueous phase concentrations of SOM and SOM-associated contaminants in the rhizosphere. In this manner, root exudates of corn were shown to disaggregate SOM of an Eutric Cambisol soil (*100*). Such a disaggreation, along with other plant-driven processes mentioned above, could lead to higher bioavailability of highly hydrophobic contaminants associated with SOM. For example, root exudates of *Cucurbita pepo* L. (zucchini) were shown to mobilize SOM-associated PCDDs and polychlorinated dibenzofurans (*102*). Extraction of a soil highly contaminated with these compounds by zucchini exudates resulted in a 4-fold higher concentration of dissolved contaminants compared to water extraction. Non-extractable residual concentrations of benzo[alpha]pyrene and its by-products (*103*), and PCP (*104*) have been found significantly lower in vegetated than non-vegetated soils.

In contrast, the bioavailability of less hydrophobic contaminants and their highly reactive intermediates could be lower in the rhizosphere than in non-rhizospheric soils. For example, after 5-days exposure of various polyaromatic hydrocarbons (PAHs) to vegetated and non-vegetated soils under oxygen-rich conditions, up to 40% of naphthalene and 30 % of phenanthrene residues (including their by-products) were associated with humic and fulvic acid fractions of vegetated soil; but only 14.6% and 7.1%, respectively, were associated with the same fractions of non-vegetated soils (*105*). No difference in the distribution of pyrene in these soils was observed. Similar distribution of intermediates in the rhizosphere of corn and non-rhizhospheric soils was reported recently for 2,4,6-trinitrotoluene metabolites (*106*). Both the predominant 2,4,6-trinitrotoluene degradation products observed in the experiment, monoaminodinitrotoluenes and hydroxyaminodinitrotoluenes, accumulated and disappeared more rapidly in rhizospheric than in non-rhizospheric soils. The total non-extractable residues in the rhizospheric and non-rhizospheric soils accounted for 40% and 28% at the end of the experiment, respectively.

The rhizosphere of terrestrial plants can affect bioavailability of organohalides in the environment. Based on the considerations and examples provided above, the following can be envisioned. Highly hydrophobic compounds, such as highly halogenated aryls and similar compounds, are more bioavailable in the rhizosphere than in bulk soils due to their enhanced mass transfer from the SOM to aqueous phase. This enhancement can proceed through, (i) solubilization of SOM and SOM-associated contaminants by root exudates, (ii) partial degradation of SOM by plant exudates causing changes in the soil architecture and release of sorbed contaminants, (iii) direct mobilization of SOM-associated contaminants by root exudates, and; (iv) other processes specific to the rhizosphere, such as solar-driven transport, enhanced soil diagenesis, etc. Incorporation of highly reactive and more hydrophilic

Downloaded by PENNSYLVANIA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch016

Downloaded by PENNSYLVANIA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch016

intermediates of organohalide degradation into SOM (in particular, into humic and fulvic acid fractions), results in a decrease of their availability for further microbial degradation. This incorporation is likely enhanced in rhizospheric environments due to, (i) increased general microbial activity, (ii) participation of plant enzymes in oxidative coupling reactions of these compounds with SOM components, (iii) elevated concentrations of phenolic and polyphenolic precursors of humic compounds in the rhizosphere, and; (iv) other plant-driven processes not yet identified. The fate of hydrophobic organohalides in rhizospheric environments depends on the ratio between the processes of mineralization and sequestration of parent compounds and their metabolites. This ratio is determined by the nature of a contaminant and its metabolites, their mass transfer kinetics between the SOM and aqueous phases, microbial degradation kinetics, and ability of rhizospheric microorganisms to access sorbed contaminants directly. The above consideration of enhanced hydrophobic organohalides bioavailability in rhizospheric environments is based on the data obtained for terrestrial plants. There is no information on the bioavailability of hydrophobic contaminants in the rhizosphere of wetland plants, but one would expect similar bioavailability as in terrestrial rhizospheres.

Examples given in this chapter, indicate extensive microbial RDE processes exist in the rhizosphere of sediment dwelling macrophytes, flooded plants, marshes and swamp vegetation, and plants growing on compacted or poorly aerated soils. RDE reactions in such environments are expected to be facilitated and enhanced compared to those in bulk soils and sediments. Comparative data on rhizospheric parameters of wetland and terrestrial plants, coupled with the optimal microbial reductive reaction parameters known from the literature, are presented in Table IV.

Evidence for Microbial Reductive Dehalogenation in Rhizospheric Environments

Studies of microbial RDE in rhizospheric environments are scarce and often incomplete. Examples of microbial dehalogenation reactions in rhizospheric environments are given in Table V. Incidentally, most of these are limited to trichloroethylene (TCE) reductive dechlorination, and were observed in the rhizosphere of wetland plants.

Narayanan *et al.* (*107*) studied the fate of two volatile alkyl halides, 1,1,1,-trichloroethane (TCA) and TCE , in the rhizosphere of alfalfa growing in sandy silt soil in a laboratory chamber. Methanogenic conditions were generated during the experiment, and a significant fraction of TCA and TCE disappeared thereafter. Small quantities of TCA and TCE migrated from the soil into the atmosphere; but no dechlorinated intermediates were found in the atmosphere.

Lee et al. (*108*) studied phreatophyte influence (using *Populus deltoides*, eastern cottonwood trees) on reductive dechlorination of TCE in a shallow aerobic aquifer. Eighteen months after planting, the concentration of dissolved oxygen in the sediments decreased beneath the trees. TCE concentrations were unchanged, indicating that the tree roots had not affected ground water geochemistry sufficiently

Downloaded by PENNSYLVANIA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch016

Table IV. **Suitability of Terrestrial and Wetland Rhizosphere Environments to Reductive Dehalogenation Reactions**

Favorable Conditions	*Rhizospheric Conditions of Terrestial Plants*	*Rhizospheric Conditions of Wetland Plants*
Anaerobic	Typically aerobic or microaerophilic; root tips most anoxic regions	Microaerophilic and anaerobic, except for root tips
Low redox potential	Various; root tips most reduced regions	Redox potential (-100 mV) or less
Electron acceptors	Primarily nitrate, organic acids, humic precursors and humic compounds	Organic acids, nitrate, humic and polyphenolic compounds, Fe (III), SO_4^{2-}, and CO_2 present
Dehalogenating microorganisms	Nitrate-reducers are abundant; dehalogenators may be present	All major groups of potential dehalogenators abundant; polyphenol- and humic-respirers promoted
Abundance of electron donors and nutrients	Electron donors and nutrients more abundant than in non-vegetated environments	Electron donors and nutrients more abundant and available than in non-vegetated environments
Bioavailability of organohalides	Hydrophobic organohalides readily available for biotransformation and reactive intermediates less available than in bulk soils.	Insufficient information available

Downloaded by PENNSYLVANIA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch016

Table V. Examples of Microbial Reductive Dehalogenation in Rhizospheric Environments

Plant System	*Compound*	*Terminal Electron Acceptor Process*	*Reference*
Alfalfa (*Medicago sativa*)	1,1,1-Trichloroethane and trichloroethene; no products reported	Methanogenic	(*107*)
Eastern cottonwood trees (*Populus deltoides*)	Trichloroethene dechlorinated to cis- 1,2-dichloroethene and vinyl chloride	Iron reducing	(*108*)
Various marsh plants	Trichloroethene dechlorinated to cis- and trans-1,2-dichloroethenes	Methanogenic	(*109*)
Common Club-rush (*Shoenoplectus lacustris*), Bulrush (*Typha latifolia*), Yellow Iris (*Iris pseudacorus*), Common Reed (*Phragmites australis*)	Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine); no products reported	Not determined	(*110*)
Cattail rhizomes (*Typha angustifolia*)	PCB: di- through tetrachlorinated PCBs dechlorinated to monochlorinated congeners. Highest dechlorination rates in rhizospheres of inactive plant.	Presumably methanogenic	(*111*)

to promote microbially-mediated RDE. However, TCE degradation occurred in ground water beneath a mature (19-year old) cottonwood tree nearby. A community of iron reducing microorganisms was responsible for RDE of TCE. Low molecular weight organic acids from root exudates and decaying roots served as electron donors and carbon sources. This community reductively dechlorinated TCE to cis-1,2-DCE and vinyl chloride. Similarly, natural attenuation of TCE during RDE in rhizospheric soils at the Savannah River site was recently reported (*109*). Microbial reductive dechlorination of TCE to cis-1,2-DCE and trans-1,2-DCE was observed under natural methanogenic conditions established on the site. However, these products represented less than 5% of the initial TCE added.

Enhanced microbial degradation of the herbicide atrazine under marsh plant system conditions was recently reported (*110*). The conditions tested were presumably anaerobic, but no details on microorganisms and TEAP conditions were given. Recently, the microbial reductive dechlorination of a PCB mixture, Aroclor 1242, in the rhizosphere of active and inactive (cut off at ground level) plants of *Typha angustifolia* (cattail rhizomes) was investigated and compared to non-vegetated soil (*111*). In all environments studied, reductive dechlorination of tetra- through dichlorinated PCB congeners was observed. No dechlorination of pentachlorinated PCB congeners occurred within 90 days. The rates of PCB dechlorination in the rhizosphere of active plants were lower than those in the non-vegetated soil. The highest rates of PCB dechlorination were observed in the rhizosphere of inactive plants. Some attempts were made to characterize the microorganisms responsible for the dechlorination observed. The abundance of *Archea* spp. was similar in all the environments studied. The levels of *Methanosarcinaceae* in both the non-vegetated and inactive plant soils treated with PCBs were higher than those in corresponding controls and in the active plant rhizosphere soil. This proliferation of *Methanosarcinaceae* could indicate its involvement in PCB dehalogenation. The authors suggested that PCB dechlorination was inhibited by the oxygen provided by active cattail rhizomes; and that PCB dechlorination was stimulated by anaerobic conditions under inactive plants and organic carbon from root decomposition. One may expect that naturally reduced rhizospheres could provide conditions favorable for PCB reductive dehalogenation.

Downloaded by PENNSYLVANIA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch016

Conclusions and Future Directions

It is evident that microbial RDE is a useful mechanism for organohalide degradation in rhizospheric environments, in particular in environments with low redox potential and oxygen concentration. Many aspects of rhizosphere RDE merit further study. Suggested areas of investigations are outlined below.

(i) Evaluate the relationships between the vegetation type, growing conditions, and the potential for RDE in the rhizosphere. Studies of sediment-dwelling macrophytes, flooded plants, marshes and swamp vegetation, are especially warranted

(ii) Characterize rhizosphere microbial communities and pure cultures responsible for RDE. Evaluate the role of cometabolic versus direct (halo-respiration) RDE.

(iii) Evaluate relationships of rhizosphere conditions (oxygen distribution, redox potential values, root exudate composition, soil organic matter structure and content) and the availability of relevant halogenated compounds to microbial transformation in the rhizosphere.

(iv) Evaluate the pathways of microbial RDE in the rhizosphere, in comparison to bulk soils and sediments.

(v) Evaluate spatial and temporal distribution of microorganisms responsible for RDE reactions in rhizospheric environments.

Successful accomplishment of these studies will promote a better understanding of the microbial RDE phenomenon and its value for transformation of organohalides in rhizosphere environments. Results of these studies will also augment our knowledge of the fate of organohalides in the environment. This may lead to new approaches for natural and engineered remediation of soils and sediments contaminated with these compounds.

Downloaded by PENNSYLVANIA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch016

References

1. Adriaens, P.; Barkovskii, A. L.; Albrecht I. D. In *Bioremediation of Contaminated Soils;* Adriano, D. C.; Bollag, J. M.; Frankenburger, W. T.; Sims, R., Eds.; Soil Science Society of America Press, Madison, WI, 1999, pp 175-212.
2. Fetzner, S.; Lingens, F. *Microbiol. Rev.* **1994**, *58*, 641-685.
3. Mohn, W. W.; Tiedje, J. M. *Microbiol. Rev.* **1992**, *56*, 482-507.
4. Sandman, E. R.; Loos, M. A. *Chemosphere.* **1984**, *13,* 1073-1084
5. Krueger, J. P.; Butz, R. G.; Cork, D. J. *J. Agric. Food. Chem.* **1991**, *39*, 1000-1003.
6. Ferro, A. M., Sims, R. C; Bugbee, B. J. *J. Environ. Qual.* **1994**, *23*, 272-279.
7. Haby, P.; Crowley, D. E. *J. Environ. Qual.* **1996**, *25*, 304-310.
8. Walton, B. T.; Anderson, T. A. *Appl. Environ. Microbiol.* **1990**, *56*, 1012-1016.
9. Crowley, D. E.; Alvey, S.; Gilbert, E. S. In *Bioremediation Through Rhizosphere Technology*; Kruger, E. 1.; Anderson, T. A.; Coats, J. R.; Eds.; ACS Symp. Ser., 664, American Chemical Society, Washington, DC, 1994, pp 20-36.
10. van der Meer, L. R.; de Vos, W. M.; Harayama, S.; Zehnder, A. J. B. *Microbiol. Rev.* **1992**, *56*, 677-694.
11 Spain, J. C.; van Veld, P. A. *Appl. Environ. Microbiol.* **1983**, *45*, 428-435.
12. Bumb, R. R.; Crummett, W. B.; Cutie, S. s.; Gledhill, J. R., Hummei, R. H.; Kagel, R. O.; Lamparski, L . L.; Luoma, E. V.; Miller, D. L.; Nestrick, T. J.; Shadoff, L A.; Stehl, R. H.; Woods, J. S. *Science* **1980**, *210*, 385-393.
13. Gribble, G. W. *Environ. Sci. Technol.* **1994**, *28*, 310A-319A.
14. Fenical, W. *Chem. Rev.* **1993**, *193*, 1673-1683.

15. Palleroni, N. J. In *Microbial Transformation and Degradation of Toxic Organic Chemicals*; Young, L. Y.; Cerniglia, C. E., Eds.; John Wiley & Sons, New York, NY, 1995, pp 31-27.
16. Steiert, J. G.; Crawford, R. L. *Biochem. Biophys. Res. Commun.* **1986**, *141*, 825-830.
17. van den Tweel, W. J. J; Kok, J. B.; de Bont, J. A. M. *Appl. Environ. Microbiol.* **1987**, *53*, 810-815.
18. Adriaens, P.; Fu, Q.; Grbic-Galic, D. *Environ. Sci. Technol.* **1995**, *29*, 2252-2260.
19. Barkovskii A. L., Adriaens P. *Appl. Environ. Microbiol.* **1996** , *62*, 4556-4562.
20. Albrecht, I. D.; Barkovskii, A. L.; Adriaens, P. *Environ. Sci. Technol.* **1999**, *33*, 737-744.
21. Holliger, C.; Schraa, G. *FEMS Microbiol. Rev.* **1994**, *15*, 297-305.
22. Bedard, D. L.; Quensen III, J. F. In *Microbial Transformation and Degradation of Toxic Organic Chemicals*; Young, L. Y.; Cerniglia, C. E., Eds.; John Wiley & Sons, New York, NY, 1995, pp 127-216.
23. Vannelli, T.; Hooper, A. B. *Appl. Environ. Microbiol.* **1993**, *59*, 528-535.
24. Adriaens, P.; Chang, P. R., Barkovskii, A. L. *Chemosphere* **1996**, *32*, 433-441.
25 Barkovskii, A. L.; Bouillant, M-L.; Jocteur-Monrozier, L.; Balandreau J. *Microbial Ecol.* **1995**, *29*, 99-114.
26. Barkovskii, A. L.; Boullant, M. L.; Balandreau, J. In *Bioremediation Through Rhizosphere Technology*; Anderson, T. A.; Coats, J. R., Eds.; ACS Symp. Ser., 563, American Chemical Society, Washington, DC, 1994, pp 28-42.
27. Lovley, D. R.; Coates, J. D.; Blunt-Harris, E. L.; Phillips, E. J. P.; Woodward, J. C. *Nature*, **1996**, *382*, 445-448.
28. Barkovskii, A. L.; Adriaens, P. *Environ. Toxicol. Chem.* **1998**, *17*, 1013-1020.
29. Fathepure, B. Z.; Boyd, S. A. *Appl. Environ. Microbiol.* **1988**, *54*, 2976-2980.
30. Krone, U. E.; Thauer, R. K.; Hogenkamp, H. P. C. *Biochem.*. **1989**, *28*, 4908-4914.
31. Holliger, C.; Schraa, G.; Stupperich, E.; Stams, A. J. M.; Zehnder, A. J. B. *J. Bacteriol.* **1992**, *174*, 4427-4434.
32. Woods, S. L.; Trobaugh, D. J.; Carter, K. J. *Environ. Sci. Technol.* **1999**, *33*, 857-863.
33. Fathepure, B. Z.; Tiedje, J. M. In *Bioremediation of Contaminated Soils;* Adriano, D. C.; Bollag, J. M.; Frankenburger, W. T.; Sims, R., Eds; Soil Science Society of America Press, Madison, WI, 1999, pp 339-396.
34. Vogel, T. M.; Criddle, C. S.; McCarty, P. L. *Environ. Sci. Technol.* **1987**, *21*, 722-736.
35. Griffin, G. D.; Cole, J. R.; Quiensen III, J. F.; Tiedje, J. M. *Appl. Environ. Microbiol.* **1992**, *58*, 409-411.
36. Mohn, W. W.; Tiedje, J. M. *Arch. Microbiol.* **1991**, *157*, 1-6.
37. Ni, S.; Fredrickson, J. K.; Xun, L. *J. Bacteriol.* **1995**, *177*, 5135-5139.
38. Löffler, F. F.; Sanford, R. A.; Tiedje, J. M. *Appl. Environ. Microbiol.* **1996**, *62*, 3809-3813.
39. Xun, I.; Topp, E.; Orser, C. Y. *J. Bacteriol.* **1992**, *174*, 8003-8007.

Downloaded by PENNSYLVANIA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch016

40. Nayler, O.; Insall, R.; Kay, R. R. *Eur. J. Biochem.* **1992**, *208*, 531-536.
41. Essac, E. G.; Matsumura, F. *Pharmac. Ther.* **1980**, *9*, 1-26.
42. Kuhn, E. P.; Suflita, J. M. In *Reactions and Movements of Organic Chemicals in Soils*; Sawhney, B. L.; Brown, K., Eds. Soil Science Society of America and American Society of Agronomy, Madison, WI, 1989, pp 111-180.
43. Criddle, C. S.; McCarty, P. L.; Eliott, M. C.; Barker, J. F. *J. Contam. Hydrol.* **1986**, *1*, 133-142.
44. Haggblom, M. M.; Janke, D.; Salkinoja-Salonen, M. S. *Appl. Environ. Microbiol.* **1989**, *55*, 516-519.
45. Shimp, J. F.; Tracy, J. C.; Davis, L. C.; Lee, E.; Huang, W.; Erickson, L. E.; Schnoor, J. L. *Crit. Rev. Environ. Sci. Technol.* **1993**, *23*, 41-77.
46. Flessa, H.; Fischer, W. R. *Plant & Soil* **1992**, *143*, 55-60.
47. Flessa, H. *Aquat. Bot.* **1994**, *47*, 119-129.
48. Pedersen, O.; Borum, J.; Duarte, C. M.; Fortes, M. D. *Marine Ecology-Progess Series* **1998**, *169*, 283-288.
49. Muramatsu, Y.; Yoshida, S.; Uchida. S.; Hasebe, A. *Water Air Soil Pollut.* **1996**, *86*, 359-371.
50. Flessa, H.; Fisher, W. R. *Z. Pflanzenernh. Bodenk.* **1992**, *155*, 373-378.
51. Armstrong, W.; Strange, M. E.; Gringle, S.; Beckett, P. M. *Ann. Bot.* **1994**, *74*, 287-299.
52. Terzenbach, D. P.; Blaut, M. *FEMS Microbiol. Lett.* **1994**, *123*, 213-218.
53. Holliger, C.; Schraa, G.; Stams, A. J. M.; Zehnder, A. J. B. *Biodegradation* **1990**, *1*, 253-251.
54. Picardial, F. W.; Arnold, R. G.; Couch, H.; Little, A. M.; Smith, M. E. *Appl. Environ. Microbiol.* **1993**, *59*, 3763-3770.
55. Petrovskis, E. A.; Vogel, T. M.; Saffarini, D. A.; Nealson, K. H.; Adriaens, P. In *Bioremediation of Chlorinated Solvents*; Hinchee, R. E.; Hoeppel, D.; Anderson, B., Eds.; Battelle Press, Columbus, OH, 1995, pp 61-67.
56. Criddle, C. A.; de Witt, J. T.; McCarty, P. L. *Appl. Environ. Microbiol.* **1990**, *56*, 3247-3254.
57. Fu, Q.; Barkovskii, A. L; Adriaens, P. *Abstr. Ann. Meet. Amer. Soc. Microbiol.*, **1999**, *99*, K-60, p 412.
58. Haider, K.; Mosier, A.; Heinemeyer, O. *Soil Sci. Soc. Am. J* **1987**, *51*, 97-102.
59. Frenzel, P.; Bosse, U.; Janssen, P. H. *Soil Biol. Biochem.* **1999** *31*, 421-430.
60. Wang, T. G.; Peverly, J. H. *Soil Sci. Soc. Am. J.* **1999**, *63*, 247-252.
61. Blaabjerg, V.; Finster, K. *Aquatic Microbiol. Ecol.* **1998**, *15*, 311-314.
62. Roden, E. E.; Wetzel, R. G. *Limnol. Ocenography* **1996**, *41*, 1733-1748.
63. van Bodegom, P. M.; Stams, A. J. M. *Chemosphere* **1999**, *39*, 167-182.
64. Vançura, V.; Kunc, F. *Soil Microbial Associations; Control of Structures and Functions*. Elsevier, Amsterdam, **1988**, 498p
65. Glaus, M. A.; Hejman, C. G.; Schwarzenbach, R. P.; Zeyer, J. *Appl. Environ. Microbiol.* **1992**, *58*, 1945-1951.
66. Gutierrez, A.; Caramelo, L.; Prieto, A.; Martinez, M. J.; Martinez A. T. *Appl. Environ. Microbiol.* **1994**, *60*, 1783-1788.

Downloaded by PENNSYLVANIA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch016

67. Blum, U.; Wentworth, T. R.; Klein, K.; Worsham, A. D.; King, L. D.; Geric, T. M.; Lyu, S. W. *J. Chem. Ecol.* **1991**, *17*, 1045-1068.
68. Kuiters, A.T.; Dennemann, C. A. J. *Soil Biol. Biochem.* **1987**, *19*, 765-769.
69. Wersshaw, R. L. *Environ. Sci. Technol.* **1993**, 27, 814-816.
70. D'Acqui, L. P.; Daniele, L.; Fornasier, F.; Radaelli, L.; Ristori G. G. *Eur. J. Soil Sci.* **1998**, *49*, 579-587.
71. Fu, S. Q.; Barkovskii, A. L.; Adriaens, P. *Environ. Sci. Technol.* **1999**, *33*, 3837-3842.
72. Hines, M. E.; Evans, R. S.; Genthner, B. R. S.; Willis, S. G.; Friedman, S.; Rooney-Vagra, J. N.; Devereux, R. *Appl. Environ. Microbiol.* **1999**, *65*, 2209-2216.
73. Stamper, D. M.; Traina, S. J.; Tuovinen, O. H. *J. Environ. Qual.* **1997**, *26*, 488-494.
74. Grunfeld, S.; Brix, H. *Aquat. Bot.* **1999**, *64*, 63-75.
75. Roden, E. E.; Wetzel, R. G. *Limnol. Oceanogr.* **1996**, *41*, 1733-1748.
76. Clays-Josserand, A.; Lemanceau, P.; Philippot, L.; Lensi, R. *Appl. Environ. Microbiol.* **1995**, *61*, 1745-1749.
77. Arth, I.; Frenzel, P.; Conrad, R. *Soil Biol. Biochem.* **1998**, *30*, 509-515.
78. Alexandre, G.; Bally, R. *FEMS Microbiol. Lett.* **1999**, *174*, 371-378.
79. Alexandre, G.; Jacoud, G.; Faure, D.; Bally, R. *FEMS Microbiol. Ecol.* **1996**, *19*, 271-278.
80. Bouillant, M. L.; Jacoud, C.; Zanella, I.; Favrebonvin, J.; Bally, R. *Phytochemistry.* **1994**, *35*, 769-771.
81. Shelton, D. R.; Tiedje, J. M. *Appl. Environ. Microbiol.* **1984**, *48*, 840-848.
82. Bouchard, B.; Beaudet, R.; Villemur, R.; McSween, G.; Lepinr, F.; Bisaillon, J.-G. *Int. J. Syst. Bacteriol.* **1996**, *46*, 1010-1015.
83. Cole, J. R.; Cascarelli, A. L.; Mohn, W. W.; Tiedje, J. M. *Appl. Environ. Microbiol.* **1994**, *60*, 3536-3542.
84. Tsuchiya, T.; Yamaha, T. Agric. *Biol. Chem.* **1984**, *48*, 1545-1550.
85. Patniak, G. K.; Kanungo, P. K.; Rao, V. R. *Microbiol. Res.* **1994,** *149*, 291-295.
86. Lynch, J. M.; Whipps, J. M. *Plant & Soil* **1990**, *129*, 1-10.
87. Donnelly, P. K.; Hedge, R. S.; Fletcher, J. S. *Chemosphere* **1994**, *28*, 981-988.
88. Bradley, R. L.; Fyles, J. W. *Soil Biol. Biochem.* **1995**, *27*, 1565-1571.
89. Gobran, G. R.; Clegg, S.; Courchense, F. *Biogeochemistry* **1998**, *42*, 107-120.
90. Focht, D. D. *Soil Sci.* **1992,** *154*, 300-307.
91. Wong, T. Y.; Graham, L.; O'Hara, E.; Maier, R. J. *Appl. Environ. Microbiol.* **1986**, *52*, 1008-1013.
92. Gilbert, E. S.; Crowley, D. E. *Appl. Environ. Microbiol.* **1997**, *63*, 1933-1938.
93. Hernandez, B. S.; Koh, S. C.; Chial, M.; Focht, D. D. *Biodegradation* **1997**, *8*, 153-158.
94. Alexander, M. *Environ. Sci. Technol.* **1991**, *25*, 1972-1973.
95. Mihelcic, J. R.; Lueking, D. R.; Mitzell, R. J.; Stapleton, J. M. *Biodegradation* **1993**, *4*, 141-153.
96. Harms, H. In *Biodegradation of Dioxins and Furans;* Wittich, R.-M., Ed.; Landes Bioscience, Austin, TX, 1998, pp 135-163.

Downloaded by PENNSYLVANIA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch016

97. VanVeen, J. A.; Kuikman., P. J. *Biochemistry* **1990**, *11*, 213-233.

98. Scheunert, I.; Schroder, P. *Environ. Sci. Pollut. Res.* **1998** *5*, 238-244.

99. Erickson, L. E.; Davus, L. C.; Narayanan, M. *Thermochim. Acta* **1995,** *250*, 353-358.

100. Nardi, S.; Reniero, F.; Concheri, G. *Chemosphere* **1997**, *35*, 2237-2244.

101. Fan, T. W. M.; Lane, A. N.; Pedler, J.; Crowley, D.; Higashi, R. M. *Anal. Biochem.* **1997**, *251*, 57-68.

102. Hüstler, A.; Marschner H. *Organohalogen Compounds*, **1994**, *20*, 31-34.

103. Banks, M. K.; Lee, E.; Schwab, A. P. *J. Environ. Qual.* **1999**, *28*, 294-298.

104. Ferro, A. M.; Sims, R. C.; Bugbee, B. *J. Environ. Qual.* **1994**, *23*, 272-279.

105. Walton, B. T.; Hoylman, A. M.; Perez, M. M.; Anderson, T. A.; Johnson, T. R.; Guthrie, E. A.; Christman, R. F. In *Bioremediation Through Rhizosphere Technology*; Anderson, T. A.; Coatş, J. R., Eds.; ACS Symp. Ser., *563*, American Chemical Society, Washington, DC, 1994, pp 82-92.

106. Kreslavski, V. D.; Vasilyeva, G. K.; Comfort, S. D.; Drijber, R. A.; Shea, P. J. *Bioremed. J.* **1999**, *3*, 59-69.

107. Narayanan, M.; Davis, L. C.; Erickson, L. E. *Environ. Sci. Technol.* **1995**, *29*, 2437-2444.

108. Lee, R. W.; Jones, S. A.; Kuniansky, E. L.; Harvey, G. J.; Eberts, S. M. In *Bioremediation and Phytoremediation: Chlorinated and Recalcitrant Compounds*; Wickramamayake, G. B.; Hinchee, R. E., Eds.; Battelle Press, Columbus-Richland., OH, 1998, pp 7263-268.

109. Brigmon, R. L.; Bell, N. C.; Freedman, D. L.; Berry, C. J. *J. Soil. Contam.* **1998**, *7*, 443-453.

110. McKinlay, R. G.; Kasperek, K. *Water. Res.* **1999**, *33*, 505-511.

111. Jerke, K. H.; Lemke, M. J. *Abstr. Ann. Meet. Amer. Soc. Microbiol.*, **1999**, *99*, N-122, p 471.

Downloaded by PENNSYLVANIA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch016

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch017

Chapter 17

Effects of Chemical Safeners on Herbicide Action and Metabolism in Plants

Ryan J. L. Ramsey, Frank L. Mena, and Gerald R. Stephenson

Department of Environmental Biology, University of Guelph, Guelph, Ontario N1G 2W1, Canada

Herbicide safeners have been shown to enhance the selective action of herbicides through a variety of mechanisms and pathways which may involve elevation of detoxification or conjugation reactions, altered transport activities in plants and perhaps antagonistic effects with the herbicide on the physiology of the plant or at the site of action. Most of the known mechanisms of herbicide selectivity in plants are known to be enhanced by one or more safeners. Corn, sorghum and to a lesser extent, wheat, have well-documented mechanisms for detoxifying herbicides through conjugation or oxidative reactions. The tolerance of these crops to a number of herbicides is increased by safeners known to enhance these systems. Most herbicide safeners are remarkably specific in their protection of one plant species, e.g. corn, without reducing the efficacy of the herbicide on weeds.

In the late 1940's, Otto Hoffman originated the concept of using chemical agents to protect crops against herbicide injury (*1*). Due to a malfunction in a greenhouse ventilation system, tomato plants (*Lycopersicon esculentum*) were accidentally exposed to vapors of 2,4-D. Hoffman observed that the epinastic responses caused by this sub-lethal dose of 2,4-D were absent on plants previously treated with the anti-auxin, 2,4,6-T (*2*). However, it was not until 1969, that Hoffman developed 1,8-naphthalic anhydride (NA) as the first chemical safener for commercial use. NA proved to be an effective seed applied safener for wheat (*Triticum aestivum*) and corn (*Zea mays*) against injury caused by the herbicides, barban and EPTC, respectively (*3*).

A few years later, Pallos *et al.* (*4*) patented dichlormid (R-25788) as a safener for carbamothioate herbicides (EPTC, butylate etc.) in maize. Dichlormid was more effective than the seed-applied safener, NA. Furthermore, since it was also selective dichlormid could be applied as a tank mix with herbicides and incorporated into the soil (*5*). Thus SUTAN+® (butylate and dichlormid) and Eradicane (EPTC and

In Pesticide Biotransformation in Plants and Microorganisms; Hall, J., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2000.

dichlormid) were developed as the first herbicides with selective safeners added to their formulations.

Benefits, Uses and Structures of Safeners

When moderately selective herbicides are applied at doses low enough to avoid crop injury, there are often some species of weeds that escape control. However, when doses are increased to effectively control more species of weeds, crop injury may occur. Safeners can circumvent this problem by providing protection to the crop from herbicide injury without reducing efficacy of the herbicide on the weeds. Although safeners are sometimes referred to as antidotes, protectants, or antagonists, "safener", is generally the most accepted term (*6*). This is because they are usually employed to prevent crop injury rather than to reverse phytotoxic effects that are already apparent. The possible benefits of safeners are listed below (*6*):

- Selective control of weeds in closely related crops
- Selective use of non-selective herbicides
- Greater flexibility in crop rotation
- Extending the use of existing herbicides to new uses in marginally tolerant crops
- Elucidation of sites of action and metabolism

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch017

Safeners can either be applied to the crop seed prior to planting (seed safeners) or to the soil or crop as a single pre-packaged mixture with the herbicide (selective safeners). They are most commonly used in crops such as sorghum, corn, wheat, and rice.

Despite Hoffman's initial discovery with tomatoes and a more recent observation by Hall and Soni (7) that clopyralid could prevent picloram injury to rapeseed (*Brassica napus*), there have been no commercial uses of safeners for herbicides in broadleaf crops. Activated carbon has been used as a protectant for broadleaf crops (*8*) but acts via non-physiological means and is not discussed here.

Thus far, safeners have been developed for herbicides from the aryloxyphenoxypropionate, chloroacetamide, sulfonlyurea, and thiocarbamate families (Figure 1). However, there are numerous other families of herbicides for which acceptable safeners have not been found. Structures of some of the more common types of safeners are shown in Table I.

Mode of Action

Early researchers in this field seemed to be searching for a single mechanism that would explain most crop-herbicide-safener combinations (*6*). This has not proven to be true. In fact, three general theories explaining the possible mechanisms of safener action have been presented (*9*):

1. A reduction in the rate of uptake and/or translocation of the herbicide
2. Antagonistic effects of the safener and the herbicide
3. Enhanced detoxification of the herbicide

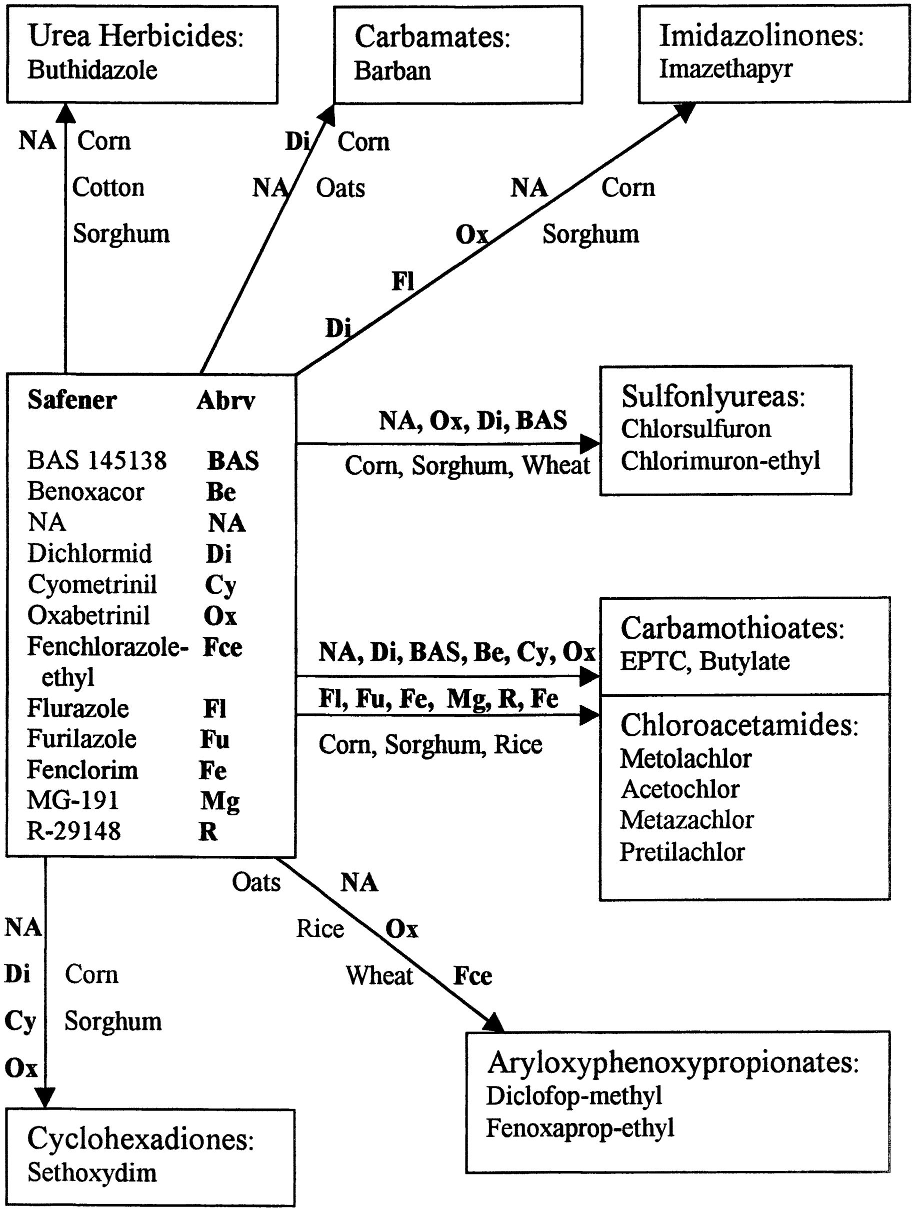
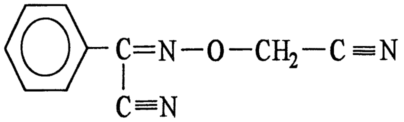
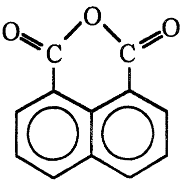
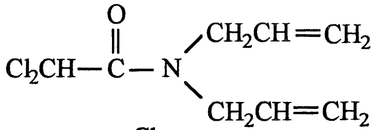
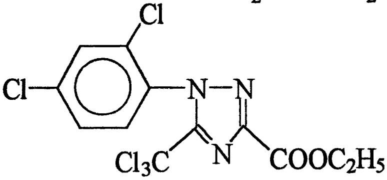
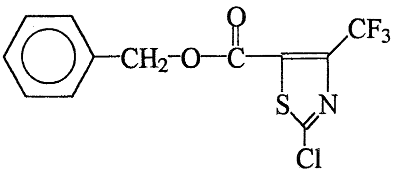

Urea Herbicides:
Buthidazole
Carbamates:
Barban
Imidazolinones:
Imazethapyr
NA
Corn
Cotton
Sorghum
Di
NA
Corn
Oats
NA
Ox
Fl
Di
Corn
Sorghum
Safener
Abrv
BAS 145138
BAS
Benoxacor
Be
NA
NA
Dichlormid
Di
Cyometrinil
Cy
Oxabetrinil
Ox
Fenchlorazole-ethyl
Fce
Flurazole
Fl
Furilazole
Fu
Fenclorim
Fe
MG-191
Mg
R-29148
R
NA, Ox, Di, BAS
Corn, Sorghum, Wheat
Sulfonlyureas:
Chlorsulfuron
Chlorimuron-ethyl
NA, Di, BAS, Be, Cy, Ox
Fl, Fu, Fe, Mg, R, Fe
Corn, Sorghum, Rice
Carbamothioates:
EPTC, Butylate
Chloroacetamides:
Metolachlor
Acetochlor
Metazachlor
Pretilachlor
NA
Di
Cy
Ox
Corn
Sorghum
Oats
Rice
Wheat
NA
Ox
Fce
Cyclohexadiones:
Sethoxydim
Aryloxyphenoxypropionates:
Diclofop-methyl
Fenoxaprop-ethyl

Figure 1. Possible herbicide/safener combinations and uses. (Modified from 11)

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch017

Soil-applied safeners and herbicides are often applied at ratios of 1:6 to 1:30 (w/w), in commercial mixtures. Unless the safeners are taken up by the plant more readily than the herbicide, one would expect very little safener to be available at the site of action in comparison to the herbicide (*9*). However, interactions between the soil and the herbicide or safener molecules may allow greater availability of the safener compared to the herbicide. A number of effective safeners structurally resemble their respective herbicide(s) and while it is possible that in these cases, the safeners may act as competitive inhibitors at the sites of herbicide action (*10*), there has

Table I. Examples of chemical structures of common types of herbicide safeners that have been used commercially.

Class	*Name/Origin*	*Chemical Structure*
Oxime ether derivative:	Cyometrinil Ciba Geigy 1978	
Napthopyranone derivative	Napthalic anhydride Hoffman, 1962	
Chloroacetamide	Dichlormid Stauffer Chem.Co. Pallos, 1971	
Phenyl Triazole	Fenchlorazole-ethyl Hoechst, 1989	
2,4-Disubstitued - 5-Thiazolecarboxylates	Flurazole Monsanto, 1980	

been little evidence to support this idea. Most research indicates that similar and dissimilar safeners induce the substrates, enzymes or cofactors necessary for their

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch017

own metabolism as well as those needed to metabolize the herbicide(s) in safened plants (*12*).With this in mind, this chapter is focussed mostly on mechanisms of safener action that influence the metabolic detoxification of herbicides in plants.

Safeners can elevate the activity of a variety of different enzyme systems in the plant such as cytochrome P450s and glutathione-S-transferases (GSTs). Among the reactions documented to date are hydroxylation, hydrolysis, glucosylation and glutathione (GSH) conjugation. The diagram below (Figure 2) illustrates possible pathways for the detoxification of xenobiotics in plants. The broken arrows in the diagram represent a proposed pathway for the glucosylation of xenobiotics in the golgi body, followed by the release of the metabolites into the apoplast via exocytosis.

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch017

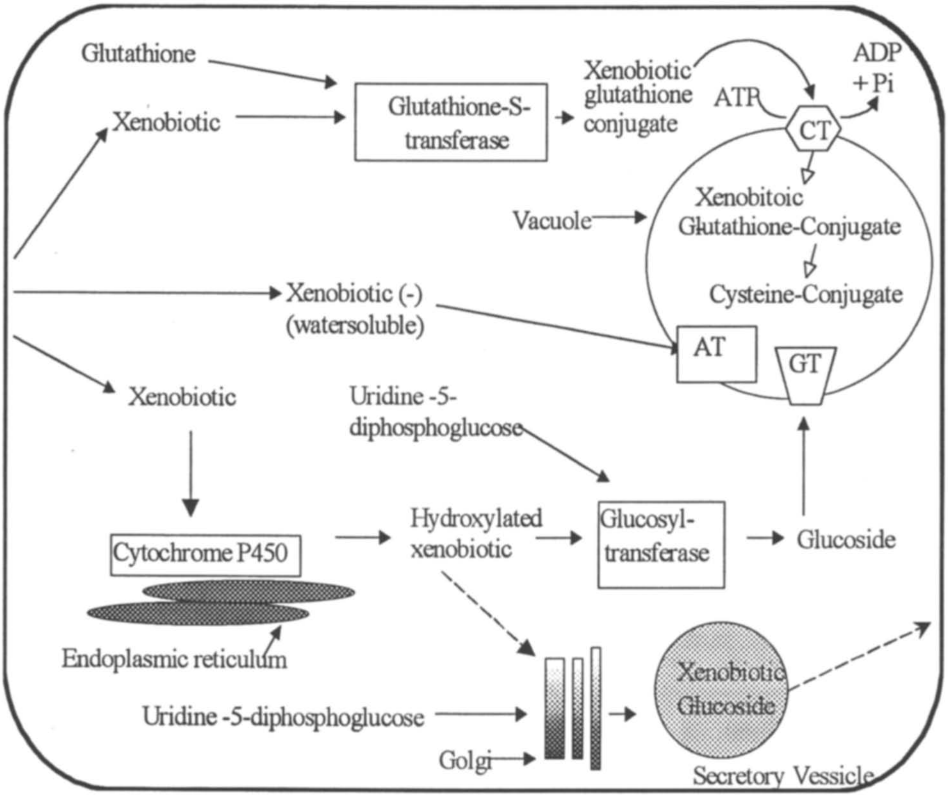

Glutathione
Xenobiotic
Glutathione-S-transferase
Xenobiotic glutathione conjugate
ATP
ADP +Pi
CT
Xenobitoic Glutathione-Conjugate
Vacuole
Cysteine-Conjugate
Xenobiotic (-) (watersoluble)
AT
GT
Xenobiotic
Uridine -5-diphosphoglucose
Cytochrome P450
Hydroxylated xenobiotic
Glucosyl-transferase
Glucoside
Endoplasmic reticulum
Uridine -5-diphosphoglucose
Golgi
Xenobiotic Glucoside
Secretory Vessicle

Figure 2. Enzyme Catalysed Reactions ofXenobiotics in Plants (Modified from 13). Abbreviations: CT, glutathione-conjugate transporter; AT, ATP–dependent xenobiotic anion transporter; GT, ATP-dependent glucoside-conjugate transporter

Mode of Action of Safeners

Effects of Safeners on Phase I Metabolism of Herbicides

Phase I metabolism involves reactions where parent compounds are either oxidized, reduced or hydrolyzed to produce a more water soluble and generally less toxic product than the parent (*14,15*). Cytochrome P450 enzymes are some of the major enzymes responsible for phase I metabolism of endogenous compounds as well as herbicides and other xenobiotics. In fact, the cytochrome P450s (P450s) are a family of catalysts that outrank any other family of enzymes in terms of the number of substrates recognized, the number of reactions catalyzed and the number of possible inducers. P450 enzymes are found in plants, bacteria, fungi, insects and mammalian tissues. They derive their name from the characteristic peak at 450 nm in the carbon monoxide IR spectrum of reduced P450. They are oxidizing catalysts that activate molecular oxygen and typically insert an oxygen atom (as a hydroxyl group) into lipophilic substrates (*16*). Their natural function is to catalyse the oxidation of many exogenous and endogenous compounds. Many P450s are also involved in the detoxification of xenobiotics and have broad substrate specificities. However, those that are involved in the biosynthesis of endogenous compounds, such as steroids and secondary plant products, have narrow substrate specificities (*16*). Some P450 enzymes are expressed constitutively while others are measurable only after induction. Even though P450s are found predominantly in microsomal membranes, some isoforms are present in other organelles. This combination of multiple isoforms, controlled by an outstanding number of inducers, makes P450s a key system for xenobiotic metabolism. It is the interplay of these two factors i.e., specific P450 forms and inducers, that determines if a xenobiotic will be metabolized by P450s in a given tissue.

Induction of P450 Monooxygenases.

Safeners are known to induce the activity of P450-dependant mono-oxygenases involved in the metabolic detoxification of chloroacetanilide, aryloxyphenoxypropionate, sulfonlyurea, imidazolinone, and sulfonamide herbicides in protected grass crops (*17, 18*). Particularly, the sulfonlyureas, imidazolinones and sulfonamides are more susceptible to attack by cytochrome P450s. These herbicides are characterized by low application doses (10-100g/ha) and these low doses may enable the rate of metabolism of the herbicide by cytochrome P450 (P450 enzymes tend to have low turnover rates) to be rapid enough to prevent phytotoxicity in the safened plant (*19*). Because of the low concentration of cytochrome P450s in plants and the inherent difficulties for their purification, our knowledge of the biochemistry and molecular biology of plant cytochrome P450 is limited (*19*). cDNA sequences encoding plant cytochrome P450s have been published (*20*). Induction of cytochrome P450 isoforms by safeners may occur via enhanced gene expression, much like that of the glutathione S-transferases (*19*). However, the exact mechanism by which safeners regulate the activity of plant P450s is currently unknown (*19*).

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch017

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch017

Cytochrome P450 Based Hydroxylation.

Until recently, the influence of safeners on cytochrome P-450 systems was unclear. Safener enhancement of oxidative metabolism *in vivo* is not necessarily accompanied by increases in total cytochrome P450 content. This may be due to the induction of P450 isozymes specific for herbicide degradation at the expense of the isozymes involved in the metabolism of endogenous substrates. Since 1990, several researchers have reported enhanced ability of microsomes extracted from monocotyledonous crop species treated with safeners, to oxidize various herbicide substrates. For example, seed treatment with the safener NA stimulated *in-vitro* hydroxylation of bentazon, metolachlor, primisulfuron, nicosulfuron, triasulfuron (*21*), chlorsulfuron (*22,23*), chlorimuron-ethyl (*24*), diclofop-methyl and chlorotoluron (*23*) and flumetsulam (*25*). In all cases, activity was consistent with cytochrome P450 being NADPH-dependent and susceptible to inhibition by known cytochrome P450 inhibitors (e.g., ABT: 1 – aminobenzotriazole, PBO: piperonyl butoxide). Furthermore, other researchers (*26*) have suggested that NA also induces cytochrome P450 mono-oxygenases responsible for the hydroxylation of imidazolinone herbicides such as imazethapyr and imazapic (AC263222).

Proof that cytochrome P450 is integral to herbicide hydroxylation has been obtained through various types of experiments. Increased hydroxylase and cytochrome b5 activity has been shown, as well as NADPH dependency in hydroxylation reactions induced by safeners. Cytochrome b5 is a component of the microsomal electron transfer reaction of cytochrome P450. In experiments performed by Davies *et al.*, (*27*), it was discovered that the total cytochrome b5 content significantly increased following seed treatment with 0.5% (w/w) NA. This suggested that NA–induced increases in cytochrome b5 would contribute to a rise in the metabolic capacity of a cytochrome P450 based herbicide hydroxylation system. Cinnamate–4-hydroxylase (a P450 enzyme) activity was identified by the appearance of p-coumaric acid in microsomes incubated with NADPH and was 40% greater in microsomes extracted from NA-treated seedlings than those extracted from untreated seedlings (*27*). Furthermore, hydroxylation of imazapic was performed by microsomes incubated with NADPH, where activity was detected by the appearance of a metabolite. Hydroxylated imazapic was not detected in control samples incubated without NADPH and/or microsomes, indicating that the metabolism was NADPH dependent. Seed treatment with 0.5% (w/w) NA significantly promoted microsomal imazapic hydroxylase activity. Incubation of microsomes extracted from NA-treated seedlings with ABT, a known cytochrome P450 inhibitor, reduced the rate of imazapic hydroxylation.

Species- Specific Cytochrome P450s.

Hinz *et al.*, (*28*) suggested it was possible for different herbicides to be metabolized by the same P450 isozymes in a particular plant species. They also suggested that different species may have different cytochrome P450s to metabolize the same herbicide. In some of their experiments, microsomes were isolated from corn, shattercane (*Sorghum bicolor*) and woolly cupgrass (*Eriochloa virillosa*) shoots that had been grown from NA treated or untreated seed to determine if metabolism of bentazon, nicosulfuron, and primisulfuron could be enhanced by NA. Their results suggested that the three herbicides may interact with the same P450 in corn and that the cytochrome P450s for primisulfuron hydroxylation are different between corn and woolly cupgrass. Furthermore, since bentazon hydroxylation in corn and shattercane microsomes were inhibited by the same cytochrome P450 inhibitor, tetcyclacis, and in woolly cupgrass there was no inhibition, they suggested that there may be a difference between these plant species in the P450s responsible for bentazon metabolism. These examples show that safeners can be used to exploit the differences in P450 metabolism of herbicides to achieve highly selective weed control.

In studies such as those mentioned above, absolute conclusions cannot be made when comparing *in vitro* microsomal activities to whole plant herbicide tolerances. It is unknown whether the same cytochrome P450s that are studied in the microsomes from NA treated tissues are responsible for *in vivo* herbicide metabolism. Broad correlation however, between the metabolism of a particular herbicide in the microsomes of a species, and the tolerance of that species to that herbicide can be made.

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch017

Effects of Safeners on Phase II Metabolism of Herbicides.

Phase II metabolism consists of conjugation of a herbicide or herbicide metabolite (often following phase I reactions) to an amino acid, glutathione (GSH) or glucose. These reactions further increase the water solubility and reduce toxicity compared to the parent herbicide molecule (*14, 15*). The enzyme(s) responsible for conjugating GSH to various substrates are the glutathione S-transferases (GSTs). GSTs and GSH are ubiquitous in aerobic organisms (*29*, *30*, *31*). Glutathione conjugation occurs in plants, animals and bacteria and performs a wide range of functions integral to survival in an oxidizing environment.

Glutathione Conjugation, Oxidative Stress and Herbicide Metabolism in Plants.

GSTs catalyze conjugation reactions with GSH by facilitating nucleophilic attack of electrophilic groups, adding GSH on to an endogenous or exogenous substrate. GSTs and GSH are known to play an important role in the detoxification of various xenobiotics in plants, and in conjunction with other substrates and enzymes such as dehydroascorbate reductase and glutathione reductase, are involved in oxidative stress management and tolerance to heat, cold and drought (*32*, *33*). The role of glutathione in oxidative stress management can be directly related to tolerance of herbicides such as the diphenyl ethers, whose mode of action involves generating reactive oxygen species. Not only can glutathione serve to detoxify herbicides through conjugation reactions, but it can also reduce some damage produced by herbicide-induced oxidative stress.

Safener Enhanced Glutathione formation.

Glutathione is a tripeptide, γ-glutamyl- cysteinylglycine, formed from glutamate, cysteine and glycine by the action of two cytosolic enzymes, γ-glutamylcysteine synthetase and glutathione synthetase. Both steps in the synthesis of GSH require energy from the hydrolysis of ATP.

An important factor that can limit GSH production is the availability of cysteine. The sulfur atom in cysteine is assimilated primarily from sulfate ions in the soil. The first two enzymes in the sulfate assimilation pathway are ATP-sulfurylase and adenosinephosphosulfate sulfotransferase (APSSTase) and both are affected by dichloroacetamide safeners (*34*, *35*, *36*). Adams *et al.,* (*34*) reported that the increase in ATP sulfurylase activity caused by dichlormid was a result of increased enzyme content in maize root and shoot tissues as opposed to the formation of an activator or removal of enzyme inhibitor molecules. Furthermore, it was noted that dichlormid increased both sulfate assimilation and GSH levels in the roots, whereas in shoots, only GSH levels were increased and sulfate levels were unaffected. An unexpected result in this same study was that two dichloroacetamide safeners for thiocarbamates in corn had little effect on sulfate levels indicating that while increased sulfate assimilation would certainly aid in GSH production reactions, it is not vital to safening activity.

Farago and Brunold (*35*) examined the induction of sulfate assimilation by dichlormid and benoxacor and found that both safeners increased the content of cysteine and glutathione in roots of maize seedlings. They found that ATP-sulfurylase activity was induced significantly only in the roots by benoxacor while APSSTase activity was induced in roots by both safeners and in the leaves only by dichlormid. Specifically, dichlormid tripled the activity of APSSTase in leaves and increased its activity 20-fold in root tissue. The increase in APSSTase activity was due to an increase in the concentration of enzyme as opposed to an alteration of effector molecules (*35*). Activity of enzymes downstream from the induced APSSTase, O-acetyl-L-serine sulfyhdrylase and sulfite reductase were unaltered by the safeners, suggesting that the effect of the safeners on sulfate assimilation was restricted to the first two enzymes in the pathway of cysteine biosynthesis. However, it is not uncommon for enzymes early in biosynthetic pathways such as APSSTase to be more highly regulated and susceptible to regulatory signals than subsequent enzymes in the pathway (*35*). APSSTase activity was elevated for 6 days after application of the safeners even though GSH and cysteine levels were high, indicating that the thiol compounds are not involved in feedback regulation of the enzyme, or that the safeners inhibited the regulatory function of these two compounds. The induction of sulfate assimilation would allow for a higher rate of GSH production through increased amounts of cysteine, which is often the limiting reagent in this process.

Regulation of GSH biosynthesis in plants is due to feedback inhibition of γ-glutamylcysteine synthetase by GSH conjugates (*37*). Safeners may interfere with the feedback inhibition of the GSH synthesis pathway resulting in continuous GSH production (*38*). Carringer *et al.,* (*39*) found that glutathione synthetase activity of maize root isolates increased *in vitro* with increasing concentrations of dichlormid, suggesting that allostearic modifications of the enzyme favouring GSH production can occur.

Non-Enzymatic Conjugation with GSH in Plants.

A number of herbicides and other substrates can undergo non-enzymatic conjugation with GSH at a measurable rate (*40*). Depending on the nature of the herbicide in question, GSH conjugation can proceed as primarily a non-enzymatic

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch017

process. A good example of this is that of fenoxaprop in wheat (*41*,*38*). Tal et al., (*38*) found that GST activity for fenoxaprop was very low in several grass crop species, yet the increase in GSH content induced by fenchlorazole-ethyl protected the plants from fenoxaprop damage, leading the authors to conclude that detoxification of fenoxaprop by GSH was primarily a non-enzymatic process.

Herbicidal substrates such as the chloroacetanilides also vary in their dependence on GST-mediated GSH conjugation with percentages of non-enzymatic conjugation ranging from 1 to 40% in corn or sorghum not treated with safeners (*42*, *43*). However, chloroacetamide safeners can increase the amount of enzymatic GSH conjugation. Ekler and Stephenson (*44*) found that the enzymatic conjugation rate (ECR) of metazachlor to GSH increased almost 5-fold in response to the safener BAS-145138. Furthermore, Ekler et al., (*43*) found that several dichloroacetamide safeners increased the amount of ECR of acetochlor from 60% to over 80%. The ratio of enzymatic to non-enzymatic conjugation of herbicides and GSH in plants varies with the herbicide and species, however in either case, safeners are able to increase both GSH content and GST activity.

These documented effects of safeners on GSH and GSH related processes certainly support the third theory proposed for safener mode of action, namely, a regulation mechanism in which the amount of herbicide reaching the site of action is reduced by enhanced herbicide detoxification. Safeners can increase GSH content and GST activity toward herbicides and prevent them from reaching their target sites in crop plants.

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch017

Safener-Enhanced GST Activity for Herbicides in Plants.

Another common mechanism for safener activity is the enhancement of GST activity in protected plants (*39*, *42*, *43*, *45*, *46*, *47*, *48*,). In plants, GSTs exist as homo- or hetero- dimers comprised of 23 to 30 kDa subunits with different subunit combinations having varying substrate affinities (*49*). Of these enzymes, GSTs in maize are the most completely characterized with five characterized and more believed to exist (*49*).

Increases in GST activity are often measured spectrophotometrically using the substrate 1-chloro-2,4-dinitrobenzene (CDNB) whose GS-conjugate absorbance maximum differs from the parent compound (*29*). However, some GST isozymes (GST II/II in particular) have limited activity towards CDNB (*49*) and determining GST activity in this manner may lead to erroneous conclusions. Recent methodologies favour the use of the ^{14}C labelled substrate of interest and chromatography techniques to quantify GST/GSH reaction products and activity (*38*, *42*, *43*, *50*).

The exact mechanism by which GST activity is increased by safener action has not yet been elucidated. However, it is more likely to be due to *de novo* enzyme synthesis (*29*) than enzyme activation. In fact, Jepson *et al.*, (*31*) reported that production of mRNA specific for a 27 kDa GST subunit (as in GST II/II) was induced by the safener dichlormid indicating that *de novo* synthesis of GSTs does occur in response to some safeners. Increases in GSH and GST activity in maize in resonse to a dichloroacetamide safener, MG-191 are shown below (Table II).

Exposure to some herbicides such as the chloroacetanilides can result in induced GST activity in plants (*42*), however, the rates of conjugation may not always be sufficient to prevent toxic effects on the plant. An interesting case is that of CDAA (N, N,-diallyl-2-chloroacetamide) which acts as both a selective herbicide and a safener, and yet can damage corn at high doses (*51*). It was one of the earliest chlroacetamide herbicides developed for selective weed control in corn, onions and other crops (*52*). Applied to corn roots, CDAA can increase GSH and GST activity (*46, 51*) but, is toxic when applied as a seed dressing (*39, 53*). Furthermore, Ezra et al., (*51*) showed that subtoxic pretreatments with CDAA increased the tolerance of corn seedlings to normally toxic doses of CDAA applied a few days later. Safeners often offer the advantage of increasing GST and GSH production in a plant at concentrations that are not phytotoxic.

In arabidopsis (*Arabidopsis thaliana*), proteins referred to as *ocs* elements affect GST expression by interacting with proteins that bind to DNA near a GST promoter region (*54*). These same *ocs* elements are related to a group of electrophile response elements found in animals. Furthermore, electrophile response elements have been found in promoter regions of rat and human GST genes (*55*). In yeast cells, electrophile-responsive elements have been implicated in oxidative stress management and gene regulation responses to this stress (*56*). Many safeners, particularly the dichloroacetamides are electrophiles and may directly interact with the electrophile-responsive elements in cells. GST induction and other effects of safener exposure may be a result of interaction with *ocs*-like elements in plant cells, which have been shown in plants to be related to GST expression and oxidative stress responses in yeast.

Table II. Effect of 24-h exposure of 2.5 day old corn seedlings to different concentrations of MG-191 on the levels of root GSH, and shoot cytosolic GST.

MG-191 (μM)	*GSH (μM·g fwt)*	*Cytosolic GST (μM/min·mg protein)*
0	0.49 ± 0.05	0.96 ± 0.11
0.1	0.48 ± 0.07	1.09 ± 0.17
1	0.77 ± 0.1	1.63 ± 0.08
10	1.03 ± 0.1	2.31 ± 0.34

SOURCE: Modified from Reference 57.

Safener induced vacuolar transporter activity.

As noted earlier, plants are essentially unable to excrete xenobiotics and must resort to sequestering them or conjugates thereof into vacuoles within the cell, or by assimilating them into cell wall material away from physiological and biochemical processes of the cell (*13*). This sequesteration of conjugates is an integral part of conjugation-mediated detoxification schemes such as those involving GSH. After primary modification of xenobiotics (usually conversion to more water soluble metabolites), sequestration can occur *via* transporter proteins on the vacuole, that are specific for anionic forms of xenobiotics, glutathione conjugates, malonyl conjugates,

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch017

glucosyl conjugates and others (*13*). Glutathione and glutathione conjugates at pH values found in the cytosol (~pH 7) will be found as anionic species owing to the two carboxyl groups of the glutatmate and glycine residues. Thus, GSH conjugates are unlikely to cross the vacuolar membrane by passive, unfacilitated means and must therefore be actively accumulated by an ATP-driven reaction or by an antiporter protein powered by an ATP-dependent ion concentration gradient (*13*). Martinoia *et al.,* (*58*) discovered an ATP-dependent vacuolar transporter protein in isolated barley (*Hordeum vulgare*) vacuoles that is specific for glutathione conjugates. In addition, at least one other type of GSH conjugate transporter has been found in plants (*59*). Both of these transporters may belong to a superfamily of ATP-binding cassette (ABC) transporter proteins (*13, 60*).

In a study of isolated barley vacuoles, Gaillard *et al.,* (*61*) found that a safener, cloquintocet-mexyl which is used in durum wheat and barley crops with clodinafop-propargyl, a herbicidal ACCase inhibitor, increased vacuolar uptake of glutathione and glucose conjugates. Vacuolar uptake of a glutathione conjugate of metolachlor and hydroxyprimisulfuron-glucose conjugate was increased by 1.89- and 2.16-fold, respectively, upon exposure to 10mM cloquintocet-mexyl. The uptake of the conjugates was found to be ATP dependent, which fits with the model of conjugate transporter enzymes involved in safener-induced uptake proposed by Coleman *et al.,* (*13*). Since isolated vacuoles were used to investigate this phenomenon (*61*), the cellular machinery required for *de novo* synthesis of transporter molecules would have been absent, leaving enzymatic modification as the only possible explanation for the increased uptake of conjugates that was observed. Upon investigation of the kinetic parameters of the transporter enzyme, it was found that the *Vmax* of the enzyme nearly doubled (2.4 to 4.9 nmol X 10^{-7}) while the *Km* remained unchanged. It was subsequently suggested that the increased uptake of conjugates may be due to improved incorporation of the enzyme into the vacuolar membrane since the unchanged *Km* indicates that the enzyme itself was unchanged (*61*).

Sánchez-Fernández *et al.,* (*60*) studied expression patterns of mRNAs coding for three different ABC proteins in *Arabidopsis thaliana* in response to exposure to varying stresses and several herbicide safeners. Benoxacor, cloquintocet, fenchlorazole, flurazole and oxabetrinil induced production of all three mRNAs, with benoxacor and oxabetrinil producing a 4-fold increase in AtMRP3 (m-RNA coding for an ABC protein) levels (*60*). Safeners can influence both the activity of existing membrane transporter proteins and the expression of genes coding for membrane transporter proteins.

The fact that safeners can affect three aspects of GSH mediated detoxification may indicate that the site of action of safeners may be a central stress response system, whereby a cascade of events prepares the plant to resist oxidative stress from various causes. It is a beneficial coincidence that the processes of this system also protect a number of crop plants against other electrophilic xenobiotics such as herbicides and that this system does not appear to occur at effective levels in very many weeds however. Ezra and Gressel (*45*) reported that while dichlormid increased sulfate assimilation and GSH content in milo pigweed (*Amaranthus paniculatus*) and redroot pigweed (*Amaranthus retroflexus*), it did not protect these species from thiocarbamate (EPTC) injury. The explanation was that although the GSH levels in

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch017

redroot pigweed were increased 2.3-fold (the same as in corn), the total level of GSH in the pigweed plants treated with the safener was still less than that found in corn prior to safener application. The fact that processes in weed species can be affected by safeners as in crop plants, yet still remain susceptible to the herbicide in question may indicate that several biochemical events are required for herbicide safening, and not all are induced to effective levels in weed species.

Other Enzymes Affected by Safeners.

In cases of oxidative herbicide metabolism by plants involving aryl or alkyl hydroxylation, some of the hydroxylated products are known to undergo a rapid conjugation with glucose. In one example, Lamoureux and Rusness (*62*) demonstrated that the safener BAS 145138 partially protected maize from chlorimuron-ethyl injury by increasing the rate of herbicide hydroxylation, glucosylation and glutathione conjugation (Figure 3). A 6-fold increase in the concentration of the o-glucoside was observed in response to treatment with BAS 145138. This increase may have been due to the changes in the glucosyl-transferase system, or as a result of more hydroxylated herbicide being available for glucosylation. Moreover, the rate of glucosylation almost doubled as a result of treatment with the safener. Kreuz *et al.* (*63*) also reported that the safener cloquintocet–mexyl enhanced the rate of hydroxylation and O–glucosylation reactions involved in the metabolism of the aryloxyphenoxypropionate herbicide CGA 184927 in wheat. However, detailed studies on the activation of UDP-glucosyl transferases by safeners are not yet available. Hatzios (*9*) suggested that the safener, NA, may also protect maize against injury from the sulfonylurea herbicide thifensufuron-methyl by inducing the activity

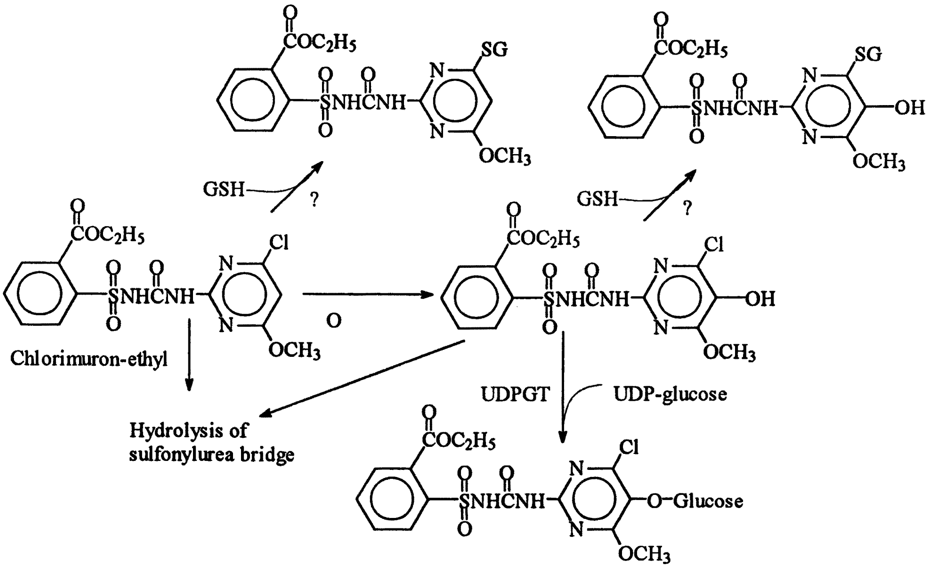


Figure 3. BAS 145138 (BAS) enhances the metabolism of chlorimuron-ethyl by hydroxylation and by conjugation with glutathione and glucose. (Modified from 62.)

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch017

of hydrolytic enzymes such as carboxyesterases. The de-esterification of thifensulfuron-methyl to it's parent acid, thifensulfuron was enhanced by 30 to 50% in maize coleoptiles when treated with NA. Furthermore, the de-esterification of fenoxaprop-ethyl to the active fenoxaprop acid was increased in wheat, barley and crabgrass (*Digitaria ischaemum*) following exposure to the safener, fenchlorazole-ethyl (*64*). Another example is that of isoxaflutole, a proherbicide that is metabolically activated to a phytotoxic diketonitrile metabolite (DKN) in most plants. Moderately tolerant plants such as maize detoxify DKN by hydrolysis (*65*). In recent studies it has been shown that maize tolerance to isoxaflutole can be enhanced by chloroacetamide safeners such as R29148 and benoxacor (*66,67*). In most of these cases however, studies of safener effects on isolated enzymes have not been conducted.

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch017

Antagonism and Altered Uptake/Translocation as a Mechanism of Action of Herbicide Safeners

The first theory of safener action mentioned in this review was that of reduced uptake or translocation of the herbicide in the crop plants. Most of the work relating to this theory has involved soil applied herbicides such as the carbamates and chloroacetanilides in monocotyledenous crops. However, evidence thus far has been contradictory with reference to this hypothesized action of safeners. Several authors (*68*, *69*,*70*, *71*) have all reported reduced herbicide uptake following treatment with a safener. However, there are also reports of increased herbicide uptake following safener treatment (*42*, *43*, *72*, *73*). Further, Kruez et al., (*74*) reported no differences in uptake of soil applied herbicides following safener treatment.

Altered translocation of herbicides following safener treatment has been reported with reference to NA and the imidazolinone herbicides imazethapyr and imazapic (*12*, *26*, *75*). Davies et al., (*12*) observed that a 0.5% seed dressing of NA significantly protected maize from imazapic injury and significantly reduced the translocation of imazapic out of maize roots. As previously stated, NA stimulates the cytochrome P450-mediated hydroxylation of imazapic by maize root and shoot tissue (*76*). Since the hydroxylated metabolite of imazapic is also a potent acetolactate synthase inhibitor, enhanced metabolism alone could not account for the ability of NA to reduce herbicide injury in this case. Davies et al., (*12*) suggested that with the increased polarity of imazapic following hydroxylation, it would be less able to penetrate membranes limiting its translocation relative to the parent molecule, citing work by Little and Shaner, (*77*) on relative mobility of hydroxylated and parent imidazolinones.

Reduced herbicide uptake may also be attributed to indirect effects of safeners. For example, the safener may prevent herbicidal effects on cuticular integrity, which may decrease transpiration and reduce herbicide uptake and translocation in safener treated plants (*78*). Davies *et al.* (*12*) also suggested that safeners may influence membrane permeability. With the accumulation of weakly acidic molecules, such as the imidazolanones, against a concentration gradient, the activity of proton-ATPases are required for the maintenance of pH gradients across membranes. Safeners, such as NA, may therefore interfere with respiratory processes causing a reduction in uptake. Other safeners, such as the oxime ethers, have been shown to inhibit the respiration of germinating sorghum seedlings (*79*) and to interfere with numerous metabolic processes such as photosynthesis (*47*).

Stephenson et al., (*80, 81*) synthesized numerous analogues to examine the structure activity relationships for chloroacetamide safeners and carbamothioate herbicides. Since structurally similar chloroacetamides were usually as highly active as safeners, the safener may act to compete with the carbamothioate herbicide at a site of toxic action, giving rise to the theory of competetive antagonism for safener action (80,81). However, since the sites of action of the carbamate and chloroacetanilide herbicides remain unknown, there has been little work to directly assess this theory.

Computer-aided molecular modeling has confirmed the similarity of several safener/herbicide combinations such as dichlormid/EPTC, Flurazole/alachlor, and fluxofenim/metolachlor in terms of charge size, shape, distribution, and connectivity indices (*82*). Walton and Casida's (*83*) discovery of a protein that binds both dichloroacetamide safeners and chloroacetanilide herbicides offered some support for the competitive antagonism theory, even though the nature of the protein is not yet fully characterized (*84*). In the case of dichlormid and EPTC, the safener binds to the protein much more tightly than the herbicide. However, with NA and EPTC, another successful safener combination, the herbicide binds to the protein more tightly than the safener by two orders of magnitude. Also, the protein discovered by Walton and Casida (*83*) may have nothing to do with the active site of the chloroacetanilides and may be a protein unrelated to their mode of action yet coincidentally bind to these compounds.

While there is some evidence for reduced uptake and translocation of herbicides caused by safeners, this does not appear to be a widely important mechanism for safener action. Similarly, there is some evidence in support of competitive antagonism, but until the sites of action of the chloroacetanilides and carbamate herbicides are found, this theory cannot be proven or refuted.

Differential effects of safeners on herbicide metabolism in crops and weeds

The most prominent question associated with safener mode of action is "why do the safeners not protect the weeds?" In the case of safeners that are applied directly to the crop seed, the weeds are not exposed to the safener and any subsequent protective effects that the safener may have had. Another scenario, seen with the foliar applied safener fenchlorazole-ethyl (FCE) is that safeners act through exploiting metabolic differences between crop and weed species, which enhance the ability of the crop plant to tolerate the herbicide while offering little or no benefit to most weed species. The best case of this is FCE which is both a safener for fenoxaprop on wheat and barley and a synergist with fenoxaprop in a number of grassy weeds (*85*). Fenoxaprop-ethyl (FE) is a herbicide used in broadleaf crops for control of weeds such as wild oat (*Avena fatua*), crabgrass, panicum (*Panicum spp.)*, foxtails (*Setaria spp.)*, and barnyardgrass (*Echinochloa spp.)* (*86*). The mode of action of this herbicide is inhibition of acetyl-CoA carboxylase (ACCase) activity in the

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch017

chloroplast, thus preventing the formation of fatty acids (*87*). Although FE is used to selectively control grass weeds in broadleaf crops, it may be used in rye, wheat, and barley if combined with the safener, FCE.

Wheat, barley and tritcale, all have substantial amounts of GSH and cysteine compared to some grassy weed species (Table 3) and given that FE is metabolized through a GSH dependent pathway (*38*), these non-protein thiol levels can predict tolerance to FE. Even though GSH and cysteine are present at detectable levels, the plants are not fully protected from FE damage. Yaacoby et al., (*64*) found that application of FCE increases GSH content in wheat and barley, but not in crabgrass. This results in an increase in the difference in GSH mediated metabolic capacities between the crop and weed species.

Furthermore, FCE appears to have two fundamental effects with respect to FE metabolism in wheat, barley, and crabgrass. The rate of de-esterification of FE to fenoxaprop (F) is increased in all three species, while measurable increases in GSH levels occurred only in wheat and barley (*38*). The former point is significant since fenoxaprop is 100-fold more potent as an ACCase inhibitor than is fenoxaprop ethyl (*87*). Thus, the plants of all three species were exposed to higher than normal levels of the toxic form of fenoxaprop yet only the barley and wheat had sufficient GSH and GST activity to convert the fenoxaprop to non-toxic metabolites. The result is a synergistic interaction between FCE and FE in the weedy grasses and an antagonistic interaction between these same two chemicals in barley and wheat (*88, 85*).

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch017

Table III. Concentrations of GSH and cysteine in shoots of several grass species.

Species	*Glutathione*	*Cysteine*	*Reference*
	(nmol·g^{-1}fresh weight)		
Sorghum	598	NA	*42*
Maize	495 ± 23*	-	*43*
Barley	126 ± 21	72 ± 10	*38*
Triticale	121 ± 12	71 ± 7	*38*
Wheat	119 ± 15	80 ± 4	*38*
Yellow foxtail	26 ± 3	12 ± 2	*38*
Oat	5 ± 2	ND	*38*
Wild oat	3 ± 2	ND	*38*
Barnyardgrass	Trace	ND	*38*
Large crabgrass	Trace	ND	*38*

Note: *Nonprotein thiol levels were measured and referred to as 'mainly glutathione'.

Molecular Approaches in Safener Research

Safeners have been used as inducers of gene expression. This control of gene expression in plants may be useful commercially to prevent herbicide injury in crops. Furthermore, controlled gene expression in plants may facilitate the study of biochemical mechanisms and help to elicudate the active sites of herbicides. Much of the current work with safeners is focused on using cDNA clones. Barrett et al. (*89*), isolated full length cytochrome P450 cDNA sequences from corn shoots and expressed these sequences in a yeast expression system. The microsomes prepared from this transformed yeast hydroxylated the herbicide bentazon. Barrett's group is continuing attempts to elevate the expression levels of this gene in yeast and to assay the effects of alternative pesticides and natural substances on the enzyme.

Safener-responsive genes have been studied by Hershey *et al.* (*20*) in the interest of creating plant gene expression systems. cDNA clones, designated In2-1 and In2-2, were isolated from poly(A)+ RNA species that were induced by the substituted benzenesulfonamide safener, *N*-(aminocarbonyl)-2-chlorobenzenesulfonamide, in maize. Further study on the two cDNA's In2-1 and In2-2 revealed that the In2-1 sequence showed some GST characteristics while no significant homology was reported for the In2-2 clone. The varying responses of the In2-1 and In2-2 mRNA's to different safeners indicated alternate mechanisms for their induction (*31*). Northern blot analysis showed that both mRNA's were rapidly and highly induced in root and leaf tissues after safener treatment, whereas no background transcript was detected in the absence of safeners.

Gene expression of a reporter gene (β-glucuronidase) in *Arabidopsis thaliana* has been examined using the In2-2 promoter activated by benzenesulfonamide herbicide safeners (*90*). It was found that In2-2 promoter activity could be induced by several safeners and the sulfonylurea herbicide chlorsulfuron. In a sulfonylurea–resistant background (in which herbicide resistance is derived from acetolactate synthase activity) induction of the In2-2 was found to be lower. Furthermore, inhibitors of branched chain amino acid synthesis (known ALS inhibitors) also induced In2-2 promoter activity. From this, the suggestion was made that there may be a correlation between In2-2 expression and inhibition of ALS activity.

Walton and Casida's (*83*) discovery of a safener binding protein in maize that binds dichloroacetamide safeners, chloroacetanilide and thiocarbamate herbicides may have opened a new avenue of safener research. The safener binding protein (SBP) was not altered chemically by safener binding. However, binding was saturable and reversible. SBP tended to bind with greater affinity to the safeners than the herbicides with some notable exceptions where compounds known to be safeners did not bind at all to the SBP (*83*). Further investigation of SBP by Scott-Craig *et al.* (*84*) reported that the sequence of the SBP had significant homology to a class of plant O-methyltransferase enzymes, although a highly conserved region was missing. The authors suggested that the SBP may be a non-functional enzyme, or not an O-methyltransferase at all. Overall, these findings suggest that the SBP may be the initial site in the action of some safeners, where SBP bound with a safener is involved in an induction of detoxification schemes. However, the fact that some effective safeners did not bind to this SBP indicate that there may be several biochemical schemes through which safening activity can occur. Further investigation into SBP to confirm its role in safening responses may lead to recombinant SBP construction in crop plants that will be specific for certain herbicide molecules, effectively making the herbicide the safener in modified plants. This idea is reminiscent of the findings of Ezra et al., (*51*) where a low, subtoxic dose pretreatment of CDAA protected corn from a future herbicidal application of the same compound. Engineering of safener

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch017

inducible systems in this manner could create physiological differences between crop plants and weeds to allow herbicide safening. This would eliminate the need to expoit naturally occurring differences in metabolism as is presently necessary to produce effective herbicide-safener interactions.

Summary and Conclusion

Most applications of herbicide safeners have been in crops such as corn, sorghum, wheat or barley, which have highly evolved stress management mechanisms. This has permitted the exploitation of metabolic differences between the crops and weeds to enhance herbicide selectivity. These safener compounds provide protection to the crop against herbicide damage through a variety of mechanisms and pathways by use of enhanced enzymatic systems and changes in transport activities within the plant. These mechanisms may involve cytochrome P450, GST/GSH, and other enzymes via hydroxylation, glucosylation and conjugation reactions. The mode of action of a safener can vary from species to species. With most successful safener/herbicide combinations, the response of the crop to the safener is different from that in the target weed, or at least the magnitude of the response is very different. A summary of the safeners presented in this paper and the biochemical effects they can have in plants is presented below (Table 4). These differences allow the control of weeds in closely related crops.

It is obvious from this review that it is not unusual for a safener to have a number of different biochemical effects that can all reduce crop injury from a particular herbicide. It is likely that the multiple mechanisms of safener action with respect to herbicide metabolism will become even more apparent with future research. Safeners do not have to induce all relevant biochemical events in order to produce an acceptable safening response. For example, not all safeners that enhance GSH conjugation have effects on sulfate assimilation and GSH production. Thus, safening activity must be considered as a whole plant/whole cell process where one or more processes are involved in producing a safening response.

Of the three proposed mechanisms of safener action, research on safener effects on herbicide metabolism and detoxification is best represented in the literature. Reduced translocation of herbicides and antagonistic effects as a result of safener exposure are not as well documented. The fact that some chloroacetamide safeners can affect cytochrome P450 metabolism of herbicides as well as three aspects of GSH mediated herbicide detoxification, suggests that safeners may act on a central stress response system, whereby a cascade of events prepares the plant to reduce oxidative stress from various causes. If this is true, it is a beneficial coincidence that the processes of this system can protect crop plants against various oxidative stress generating compounds as well as other electrophilic xenobiotics such as herbicides. This system does not appear to occur at effective levels in very many weeds however.

Current and undoubtably future endeavours with regard to safener research will focus on molecular approaches and perhaps on genetically modified plants. The question might then be asked, 'Are safeners redundant?' Why not genetically modify

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch017

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch017

Table IV. Mechanisms of herbicide safeners in plants

Safener	*Altered uptake, translocation, sequesteration*	*Phase 1 Metabolism*		*Phase 2 Metabolism*				*Antagonism*	*Other*	
		Hydroxylation Cytochrome P450	*Hydrolysis*	*Increased GST activity and GSH conjugation*	*Increased GSH production*	*Induced non-enz GSH conjugation*	*Increased rate of O-glucosylation*		*Increased sulfate assimilation*	*Increased mRNA production*
NA	*12*	*91, 27*	*9*					*68, 92*		
Dichlormid	*43*	*93*		*94*	*39*	*17*		*3*	*34*	
MG-191	*43*	*95*		*57*	*57*					
Benoxacor		*21,96*		*97*	*35*				*35*	*60*
Bas 145138	*43*	*62*		*62*	*98*		*62*			
Flurazole				*98*	*98*			*99*		*60*
Fenchlorazole-ethyl			*64*	*100*	*85*	*85*				*60*
Oxabetrinil	*72*	*21*		*82*	*82*	*82*		*99*		*60*
Fluxofenim	*72*	*21*		*82*	*82*	*82*				
Cloquintocet-mexyl	*61*	*62*		*101*			*63*			*60*

Numbers refer to data source.

the plant to be able to tolerate herbicide injury, thereby eliminating the need for safeners? The transgenic approach to alter or increase the levels of target site enzymes is a good approach if the goal is to increase crop tolerance to herbicides like glyphosate that are only metabolized to a low degree in plants. However, when metabolic detoxification systems and inducers (safeners) of these sytems are clearly known, it becomes quite feasible to use existing safeners or to develop safeners to enhance crop tolerance to new herbicides, once it is known that they are detoxified by inducible systems. There is now a long history to confirm the safety and effectiveness of this approach for providing highly selective chemical weed control and thus safeners will continue to have a niche in the agrochemical industry even with the acceptance of genetically engineered crops.

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch017

References

1. Hoffman, O. L. *Weed Sci.* **1962**, *10*, 322-323.
2. Hoffman, O. L. In, *Chemistry and Action of Herbicide Antidotes*; Pallos, F. M.; Casida, J. E. Eds. Academic Press: NewYork, NY, 1978.
3. Stephenson, G. R.; Ezra, G. *Proc. Br. Crop Prot. Conf. Weed.* 1982, 451-459
4. Pallos, F. M.; Brokke, M. E.; Arneklev, D. R. 1972. *Belgian Patent 7821120*
5. Chang, F. Y.; Bandeen, J. D.; Stephenson, G. R. *Can. J. Plant Sci.* **1972**, 707-714
6. *Crop Safeners for Herbicides*; Hatzios, K.K.; Hoagland, R. E., Eds.; Academic Press: San Diego, California, 1989, 3-8.
7. Hall, J. C.; Soni, M. *Pestic. Biochem. Physiol.* **1989**, *33*, 1-10.
8. Locascio, S. J. *Proc. South. Weed Conf.* **1967**, *20*, 157-163.
9. Hatzios, K.K. In *Regulation of Enzymatic Systems Detoxifying Xenobiotics in Plants;* Hatzios, K. K. Ed.; 1997. NATO ASI Series Partnership Sub-Series. 3. High Technology; Kluwer Academic Publishers: Dordrecht, The Netherlands, 37. pp 275-288.
10. Chang, F. Y.; Bandeen, J. D.; Stephenson, G. R. *Weed Res.* **1973**, *13*, 399-406.
11. Devine, D. M., Duke, S. O.; Fedtke, C. *Physiology of Herbicide Action*; Prentice Hall: Englewood Cliffs, NJ, 1993; pp 376-394.
12. Davies, J.; Caseley, J. C.; Jones, O.; Barrett, M.; Polge, N. *Pestic. Sci.* **1997**, *52*, 29-38.
13. Coleman, J.; Blake-Kalf, M.; Emyr-Davies, T. *Trends Plant Sci.* **1997**. *2,4,* 144–156.
14. Williams, R. T. Detoxification Mechanisms 2nd ed. Chapman and Hall, Ltd. London, England. 1959; 796p.
15. Shimabukuro, R. H. In *Weed Physiology; Duke*, S. O., Ed.; Herbicide Physiology Volume II. CRC Press: Boca Raton, FL, 1985; pp 216-217.
16. Halkier, B.A. *Phytochemistry.* **1996**, *43*,1, 1-21.
17. Leavitt, J. R. C.; Penner, D. *J. Agric. Food Chem.* **1979**, *27*, 533-536.
18. Kreuz, K.; Tommasini, R.; Martinoa, E. *Plant Physiol.* **1996**, *111*, 349-353.
19. Durst, F.; O'Keef, D. P. *Drug Metab. Drug Interac.* **1995**, *12*, 171-186.
20. Hershey, H. P.; Stoner, T. D. *Plant Mol. Biol.* **1991**, *17*, 679-690.
21. Moreland, D.E.; Corbin, F. T.; McFarland, J. E. *Pestic. Biochem. Physiol.* **1993**, *47*, 206-214.

22. Frear, D. S.; Swanson, H. R.; Thalacker, F. W. *Pestic. Biochem. Physiol.* **1991**, *41*, 274-287.
23. Zimmerlin, A.; Durst, F. *Plant Physiol.* **1992**, *100*, 874-881.
24. Polge, N.D., Barrett, M. *Pestic. Biochem. Physiol.* **1995**, *53*, 193-204.
25. Frear, D. S.; Swanson, H. R.; Tanaka, F. S. *Pestic. Biochem. Physiol.* **1993**, *45*, 178-192.
26. Barrett, M.; Maxson, J. M. *Z. Naturforsch.* **1991**, *46*, 897-900.
27. Davies, J.; Casely, J. C.; Jones, O. T. G.; Barret, M.; Polge, N. D. *Pestic. Sci.* **1998**, *52*, 29-38
28. Hinz, J. R. R.; Owen, M. D. K.; Barrett, M. *Weed Sci.* **1997**, *45*, 474-480.
29. *Molecular Toxicology;* Josephy, P. D. (Ed.); Mannervik, B.; de Montellano. P. O. Oxford University Press: New York, NY, 1997; p 152-169.
30. Droog, F. *J. Plant Growth Regul.* **1997**. *16*, 95-107
31. Jepson, I.; Lay, V. J.; Holt, D. C.; Bright, S. W. J.; Greenland. A. *J. Plant Mol. Biol.* **1994**, *26*, 1855-1866.
32. Alscher, R. G., *Physiol. Plant.* **1989**, *77*, 457-464.
33. Salin, M. L. *Physiol. Plant.* **1988**, *72*, 681-689.
34. Adams, C. A.; Blee, E.; Casida, J. E. *Pestic. Biochem. Physiol.* **1983**, *19*, 350-360.
35. Farago, S.; Brunold, C. *Plant Physiol.* **1990**, *94*, 1808-1812.
36. Farago, S.; Brunold, C.; Kreuz, K. *Physol. Plant.* **1994**, *91*, 537-542.
37. Rennenberg, H. *Phytochemistry.* **1982**, *21*, 2771-2781.
38. Tal, J. A.; Hall, J. C.; Stephenson, G. R. *Weed Res.* **1995**, *35*, 133-139.
39. Carringer, R. D.; Rieck, C. E.; Bush, L. P. *Weed Sci.* **1978**, *26*, 167-171.
40. Rozman, K. K.; Klaassen, C. D. In *Toxicology: The Basic Science of Poisons.* Casarett, L; Doull J., 5th Ed.; McGraw-Hill Health Professions Division: New York, NY, 1996, pp 177-183.
41. Romano, M. L.; Stephenson, G. R.; Tal, A.; Hall, J. C. *Pestic. Biochem. Physiol.* **1993**, *46*, 181-189.
42. Gronwald, J. W.; Fuerst, E. P.; Eberlein, C. V.; Egli, M. A. *Pestic. Biochem. Physiol.* **1987**, *29*, 66-76.
43. Ekler, Z.; Dutka, F.; Stephenson, G. R. *Weed Res.* **1993**, *33*, 311-318.
44. Ekler, Z.; Stephenson, G. R. *Weed Res.* **1989**, *29*, 181-191.
45. Ezra, G.; Gressel, J. *Pestic. Biochem. Physiol.* **1982**, *17*, 48-58.
46. Lay, M. M.; Casida, J. E. *Pestic. Biochem. Physiol.* **1976**, *6*, 442-456.
47. Zama, P.; Hatzios, K. K. *Weed Science.* **1986**, *34*, 834-841.
48. Timmerman, K. P.; *Physiol. Plant.* **1989**, *77*, 465-471.
49. Dixon, D. P.; Edwards, R.; Cole, D. *J. Proc. Br. Crop Prot. Conf-. Weeds.* 1997, 759-764.
50. Tal, J. A.; Romano, M. L.; Stephenson, G. R.; Schwan, A. L.; Hall, J. C. *Pestic. Biochem. Physiol.* **1993**, *46*, 190-199.
51. Ezra, G; Rusness, G. D.; Lamoureux, G. L.; Stephenson, G. R. *Pestic. Biochem. Physiol.* **1985**, *23*, 108-115.
52. Hamm, P. C.; Speziale, A. J. *J. Agric. Food Chem.* **1956**, *4*, 518-522.
53. Chang, F. Y.; Stephenson, G. R.; Bandeen, J. D. *Weed Sci.* **1973**, *21*, 292-295.
54. Chen, W., G. Chao, and K. B. Singh,. *Plant Journ.* **1996**, *10*, 955-966.
55. Jaiswal, A. K. *Biochem. Pharm.* **1994**, *48*, 439-444.
56. Ruis, H.; Schüller, C. Stress signaling in yeast. *BioEssays*, **1995**, *17*, 959-965.

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch017

57. Dutka, F.; Kömives, T. In: *Pesticide Science and Biotechnology 6th IUPAC Congress of Pesticide Chemistry*; Greenhalgh, R.; and Roberts, T. R., Eds. 1987. pp. 201-204.
58. Martinoia, E.; Grill, E.; Tomassini, R.; Kreuz, K.; Amrhein, N. *Nature*. **1993**, 364, 247-249.
59. Hortensteiner, S.; Vogt, E.; Hagenbuch, B.; Meier, P. J.; Amrhein, N.; Martinoia, E. *J. Biol. Chem.* **1993**, *268*, 18446-18449.
60. Sánchez-Fernández, R.; Ardiles-Díaz, W.; Van Montagu, M.; Inzé, D.; May, M. *J. Mol. Gen.* **1998**, *258*, 655-662.
61. Gaillard, C.; Dufaud, A.; Tommasini, R.; Kreuz, K.; Amrhein, N.; Martinoa, E. *FEBS Letters* **1994**.*352, 2*, 219-221.
62. Lamoureux, G. L.; Rusness, D. G. *Z. Naturforsch*. **1991**, *46c*, 882-886.
63. Kreuz, K.; Gaudin, J.; Stingelin, J.; Ebert, E. *Z. Naturforsch.* **1991**, *46c*, 901-905.
64. Yaacoby, T.; Hall, J. C.; Stephenson, G. R. *Pestic. Biochem. Physiol.* **1991**, *41*, 296-304.
65. Pallet, K. E.; Little, J. P.; Sheekey, M.; Veerasekaran, P. *Pestic. Biochem. Physiol.* **1998**, *62*, 113-124.
66. Sprague, C.; Penner, K. E. *ACS Abstracts*. **1999**.
67. Scribbs, J. M; Lee, D. L. U.S. Patent 5627131, **1997.**
68. Thiessen, E. P. *Masters Thesis*, University of Guelph, Guelph, Ontario. **1978**.
69. Ezra, G.; Krochmal, E.; Gressel, J. *Pestic. Biochem. Physiol.* **1982**, *18*, 107-112.
70. Ketchersid, M. L.; Vietor, D. M.; Merkle, M. G. *J. Plant Growth Regul.* **1982**, *1*, 285-294.
71. Han, S.; Hatzios, K. K. *Pestic. Biochem. Physiol.* **1991**, *39*, 270-280.
72. Yenne, S. P.; Hatzios, K. K.; Meredith, S. A. *J. Agric. Food Chem.* **1990**, *38*, 1957-1961.
73. Fuerst, E. P.; Gronwald, J. W. *Weed Sci*. **1986**, *34*, 354-361.
74. Kreuz, K.; Gaudin, J.; Ebert, E. *Weed Res*. **1989**, *29*, 399-405.
75. Shaner, D. L. *Z. Naturforsch*. **1991**. *46c*, 893-896.
76. Davies, J.; Caseley, J. C. *Proc. Br. Crop Prot. Conf-. Weeds*. 1993, 195-200
77. Little, D.L.; Shaner, D. L. In *The Imidazolinone Herbicides*; Shaner, D. L.; O'Connor, S. L., Eds. CRC Press Boca Raton, FL. 1991. 53-69.
78. Ebert, E., *Weed Res*. **1982**, *22*, 305-311.
79. Ketchersid, M. L.; Merkle, M. G. *Proc. South. Weed. Sci. Soc.* **1983**, *36*, 386
80. Stephenson, G. R.; Bunce, N. J.; Makowski, R. I.; Curry, J. C. *J. Agric. Food Chem.* **1978**, *26*, 137-140.
81. Stephenson, G. R.; Bunce, N. J.; Makowski, R. I.; Bergsma, M. D.; Curry, J. C. *J. Agric. Food Chem.* **1979**, *27*, 543-547.
82. Yenne, S. P.; Hatzios, K. K. *J. Agric. Food Chem.* **1990**, *38*, 1950-1956.
83. Walton, J. D.; Casida, J. E. *Plant Physiol*. **1995**, *109*, 213-219.
84. Scott-Craig, J. S.; Casida, J. E.; Poduje, L.; Walton, J. D. *Plant Physiol.* **1998**, *116*, 1083-1089.
85. Stephenson, G. R.; Tal, A.; Vincent, N. A.; Hall, J. C. *Weed Tech.* **1993**, *7*, 163-168.
86. Lefsrud, C.; Hall, J. C. *Pestic. Biochem. Physiol.* **1989**, *34*, 218-227.

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch017

87. Hoope, H. H.; Zacher, J. *Pestic. Biochem. Physiol.* **1985**, *24*, 298-305.
88. Hall, J. C.; Stephenson, G. R. *Proc. Br. Crop Prot. Conf. Weed.* **1995**, 261-268.
89. Barrett, M. *National Technical Information Service, Fedrip Database*. **1998**.
90. De Veylder, L.; Van Montagu, M. Inzé, D. *Plant Cell Physiol.* **1997**, *38*, 568-577.
91. Barrett, M. *Drug Metab. Drug Interac.* **1995**, *12*, 299-315.
92. Wilkinson, R. E.; Smith, A. E. *Weed Sci.* **1975**, *23*, 90-92.
93. Frear, D. S.; *Drug Metab. Drug Interac.* **1995**, *12*, 329-357.
94. Dean, J. V.; Gronwald, J. W.; Everlein, C. V. *Plant Physiol.* **1990**, *92*, 467-473.
95. Jablonkai, I.; Hatzios, K. K. *Pestic. Biochem. Physiol.* **1994**, *48*, 98-109.
96. Fonné-Pfister, R.; Kreuz, K. *Phytochemistry.* **1990**, *29*, 2793-2796.
97. Irzyk, G. P.; Fuerst, E. P. *Plant Physiol.* **1993**, *102*, 803-810.
98. Hulesch, A.; Dutka, F. *Proc. Br. Crop Prot. Conf-. Weeds.* **1993**, 207-212.
99. Stephenson, G. R.; Ezra, G. *Weed Sci.* **1987**, *35*, 24-27.
100. Cummins, I.; Cole, D. J.; Edwards, R. *Pestic. Biochem. Physiol.* **1997**, *59*, 35-49.
101. Reichers, D. E.; Irzyk, G. P.; Jones, S. S.; Fuerst. E. P. *Plant Physiol.* **1997**, *114*, 1461-1470.

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch017

Downloaded by PENNSYLVANIA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch018

Chapter 18

Metabolism of Xenobiotics in the Rhizosphere

D. E. Crowley, E. Luepromechai, and A. Singer

**Department of Environmental Sciences, University of California,
Riverside, CA 92521**

Plant signal compounds, including salicylic acid, flavonoids, and monoterpenes, have structures that are analogous to many anthropogenic contaminants and are speculated to function as natural substrates that cause induction of cometabolic pathways for degradation of organic xenobiotics. This review examines evidence for enhanced cometabolism of xenobiotics in the plant rhizosphere, and the potential function of signal compounds for induction of degradative pathways for chlorinated phenols, hydrocarbons, polychlorinated biphenyls (PCBs) and polyaromatic hydrocarbons (PAHs). Although, not well examined, one likely mechanism for cometabolic degradation of these substances involves the induction of cytochrome P-450 monooxygenases. A previous limitation in studies on xenobiotic degradation in the rhizosphere has been the emphasis on isolating individual bacterial degrader strains. In research presented here, experimental data are shown in which a 16S rDNA based approach is used to examine enrichment of PAH-degrading microbial communities in soil exposed to monoterpenes and other signal compounds. Such an approach may be particularly useful for characterizing the effects of plant derived substances on bacterial communities and the potential for using selected plants to enhance the degradation of soil organic contaminants.

Plants produce a variety of chemical substances with structures that are analogous to those of many commercially produced chemicals. For centuries, this phytochemical diversity has been exploited as a resource for the development of pharmaceuticals and pesticides, but more recently has been recognized as a source of chemical analogs that can be used to manipulate soil microorganisms to degrade soil contaminants. Among the most structurally diverse biosynthetic compounds in nature are terpenes, alkaloids,

In Pesticide Biotransformation in Plants and Microorganisms; Hall, J., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2000.

and phenolics that function as membrane sterols, pigments, biocides, insect attractants, growth hormones, and signal compounds. To date, over 40,000 different plant terpenoids, alkaloids, and phenolics have been chemically characterized (*1*), only a few of which have been studied in relation to their biochemical ecology. Nonetheless, without exception, all of the various compounds synthesized by plants are broken down through natural processes. An understanding of the degradation pathways for natural plant substances and how plant-microbial systems can be manipulated to enhance biodegradation is directly relevant to the cleanup of environmental contaminants. In addition to the direct application of plant compounds as soil amendments, the cultivation of selected plants that release specific compounds into the rhizosphere directly to trigger degradation remains a virtually untapped technology for bioremediation.

Downloaded by PENNSYLVANIA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch018

Chemicals released by plants can beneficially affect xenobiotic degradation by at least three mechanisms, including the selective enrichment of degrader organisms, enhancement of growth-linked metabolism, and the induction of cometabolism in certain microorganisms that carry degradative genes and plasmids. One of the general benefits of using plants to assist biodegradation is the increase in population densities of degrader organisms in the rhizosphere, which are otherwise too low to achieve biodegradation of xenobiotics at an acceptable rate (*2-4*). A second benefit may be the nonselective priming of degrader organisms, referred to as growth-linked metabolism (*5,6*). With growth-linked metabolism, the xenobiotic may be readily degraded, but due to low solubility or sorption on to clay and organic matter, is not present in solution at a sufficiently high concentration to support an active degrader population. By stimulating the growth and metabolism of the degrader population with a nonspecific substrate, enzymes required for metabolism of the target compounds may be produced at higher levels. For example, 3-chlorobenzoate degraders can be enhanced by simple additions of glucose or rhizosphere exudates to soil (*6*). Lastly, cometabolism, which is the subject of this chapter, may permit the initial attack of substances that otherwise remain unrecognized as a carbon substrate by the soil microflora. In some cases, the term cometabolism has been used to refer to biodegradation of compounds that cannot be used as a carbon or energy source for growth. A broader definition of cometabolism includes the fortuitous degradation of compounds that may also be used for carbon or energy, but which do not induce the necessary enzymes when presented as a sole substrate.

To date, the majority of research on biodegradation of plant signal compounds has focused on individual microbial isolates and key enzymes, and there are surprisingly few model systems. Among the 22,000 terpenes, only a dozen or so have been studied for their biodegradation pathways, and only one compound, camphor, has been studied in detail (*7*). Moreover, these studies have focused on individual bacterial isolates, whereas most biodegradation processes are almost certainly carried out by consortia of microorganisms. Microbial consortia typically include various members that are not readily culturable or that may be lost during enrichment culture in liquid media. In addition to the challenge of monitoring these individual members and deciphering their roles, consortia also are much more difficult to manipulate, and may be rate limited by the activity of individual members. It may also be expected that various catabolic niches within a consortium may be filled by interchangeable or competing members, and that for chlorinated hydrocarbons some intermediate metabolites may accumulate that are inhibitory to complete mineralization (*8*). Thus, whether soil microbial communities that degrade xenobiotics are benefited by the presence of a plant depends on many factors including the rhizosphere competence of the degrader bacteria, the plant species, the location of degrader organisms in specific root zones, and probably even the nutritional status and health of the plant. In unraveling this complexity, new tools are available that permit community analysis after biostimulation with potential cometabolites, which permit the rapid screening and evaluation of interesting plant compounds. The objective of this chapter is to examine what is known about biodegradation of chemically diverse plant substances, the possible role of these substances for enhancing cometabolism in the plant rhizosphere and the potential for manipulating biodegradation processes through the use of selected plant-microbial systems.

Downloaded by PENNSYLVANIA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch018

Cometabolism of Xenobiotics

Xenobiotic compounds that are degraded by cometabolism include polycyclic aromatic hydrocarbons (PAHs), and a plethora of chlorinated hydrocarbons including various pesticides, polychlorinated biphenyls (PCBs), dioxins, chloroanilines, chlorophenols, and chlorinated solvents such as chloroform, trichloroethylene (TCE), and dichloroethylene (DCE) (*9*). The principle enzymes for degradation of hydrocarbons and halogenated organic compounds are oxygenases. These enzymes, which are classified as either monooxygenases or dioxygenases, generate highly reactive monoatomic or diatomic oxygen species, respectively, that react with the enzyme bound substrate. One of the primary factors of concern for bioremediation is the induction of broad specificity enzymes that are capable of binding xenobiotic substrates. One of the most highly studied and important group of monooxygenases are the cytochrome P-450 enzymes, which constitute a superfamily of heme-thiolate proteins. Cytochrome P-450s enzymes are widely distributed in bacteria, fungi, plants and animals, and are involved in detoxification and transformation of a wide variety of substances including polycyclic aromatic hydrocarbons (*10*) and haloaromatic compounds (*11*). Dioxygenases that are of particular interest for bioremediation include enzymes for degradation of toluene, PAHs, biphenyl, dioxin, and catechols. Many of these enzymes have broad substrate specificities that permit cometabolism when induced by appropriate substrates. Among the most versatile enzymes are the methane monooxygenases that are capable of metabolizing many halogenated solvents containing one or two carbon atoms. Another important example is toluene dioxygenase. When expressed in *Pseudomonas putida* NCIMB 11767, this enzyme oxidizes phenol, monochlorophenols, several dichlorophenols, biphenyl, naphthalene and a range of alkylbenzenes (*12*).

Cometabolism is thought to be relevant to the aerobic degradation of most chlorinated organic compounds, even if they cannot be used as a carbon source (*9*). After the first transformation step in a cometabolic process, chlorinated metabolites may be produced that must undergo dehalogenation before they can be funneled into common degradation pathways. One relevant example is the induction of enzymes for degradation of polychlorinated biphenyls after growth on biphenyl, the nonchlorinated analog of these compounds. Following ring cleavage, the metabolites generated from the upper pathway include chlorobenzoates that can serve as carbon and energy sources for the degrader or other microorganisms in the vicinity. Chlorobenzoates in some cases may accumulate, or are degraded by dehalogenation. This may occur anaerobically using an aryl dehalogenase (*13*), or aerobically by the chlorocatechol pathway via a diooxygenase (*14*). For other chlorinated xenobiotics, dechlorination also may occur by spontaneous dechlorination during various oxidation steps, or via glutathione-dependent or coenzyme A-dependent dehalogenases. In some cases, peroxidases also have been shown to carry out dechlorination reactions on various aryl halides such as chlorobenzenes.

Monooxygenase enzymes that have been intensively studied in relation to cometabolism include methane monooxygenase and monoxygenases for toluene and xylene. In the case of chlorinated solvents such as TCE, degradation can occur via monooxygenases after induction of methanotrophic bacteria by growth on methane. Chlorinated solvents can also be degraded by some ammonia oxidizing bacteria that possess ammonia monooxygenase (*15*). In a new strategy aimed at deliberate construction of pathways in single organisms, biodegradation of chlorinated solvents combines a cytochrome P-450 monooxygenase with toluene dioxygenase, which is capable of dehalogenation of chlorinated C2 compounds. Using this approach, Logan and coworkers, constructed a *P. putida* strain which expresses P-450$_{CAM}$ to carry out reductive dechlorination of pentachloroethane (*16*). The product of this reaction then undergoes oxidative dechlorination by the toluene dioxygenase system for oxidative dechlorination, which leads to complete mineralization.

Rhizosphere effects on biodegradation of xenobiotics have been studied for a variety of compounds, although the mechanisms by which certain plants enhance biodegradation are still poorly understood (*4*). In some cases, the rhizosphere effect may be attributed simply to an increase in the numbers of degrader organisms, without an increase in the ratio of degrader to nondegrader organisms (*3,6*). Population increases occur as a result of carbon inputs from rhizodeposition of organic substances from living roots, or may occur after the death and turnover of the roots. Individual plants may have root lengths of several miles, with half or more of their biomass underground. So far, most research on phytoremediation has used a few model plant species that have been found to work by trial and error or that are convenient, while other studies have attempted to elucidate the so-called “rhizosphere effect”. Commonly selected plants include densely rooted, fast growing grasses and plants such as *Brassica* sp. with fine root systems. Certain tree species, such as mulberry (*Morus alba* L.) and poplar (*Populus deltoides*), also have been found to be beneficial for enhancing bioremediation, and are being promoted for phytoremediation of chlorophenols and chlorinated solvents such as TCE (*17*). However, there are many thousands of untested plant species that may produce compounds that are beneficial for the selective enrichment of degrader bacteria, or for inducing cometabolism of xenobiotics.

Downloaded by PENNSYLVANIA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch018

Phytochemicals of interest for bioremediation.

Among some of the most diverse chemicals produced by plants are flavonoids, terpenes, alkaloids, and phenolics that comprise some 40,000 different known compounds. To date, 22,000 different terpenoids have been described (*18*). In addition to these compounds, 10,000 different alkaloids have been chemically identified, as well as 8,000 different phenolics, the latter including flavonoids (*19*). Most of these compounds have only been cursorily examined to determine their structures, and their biodegradation pathways are almost completely unknown.

Downloaded by PENNSYLVANIA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch018

Plant Terpenoids

Terpenes are the largest single class of natural products, and are based on polymerization of 5-carbon isoprene units. The central pathway involves synthesis of hydroxymethylglutaryl coenzyme A from acetate, leucine, and other sources (*20*). This precursor is converted to mevalonic acid, and then undergoes decarboxylation and phosphorylation to yield a compound called isopentenyl pyrophosphate (*21,22*). This 5 carbon unit is polymerized into compounds with 10, 15, 20 or more carbons, that undergo modifications to yield the final compound. Monoterpenes consist of 2, 5-carbon isoprene units, with sesquiterpenes, diterpenes, and triterpenoids containing 3, 4, and 6 isoprene units, respectively.

Essential terpenoids include membrane sterols, carotenoids, ubiquinone, and steroid hormones (*20*). Terpenes also serve as natural fragrances, sex hormones of flowers and insects, growth hormones, color pigments, and as electron acceptors for shunting excess photons out of the thylakoids during photosynthesis. They also may function as storage compounds that are metabolized during drought (*23*). Terpenes with signal functions include compounds that control insect herbivory, pollination, phytoalexins for defense against pathogens, and allelopathy. In an example of interplant signaling, myrcene, a volatile terpene produced by cotton (*Gossypium hirsutum L.*) plants, is released during herbivory to trigger synthesis of insecticidal terpenoid aldehydes in adjacent plants. Certain terpenes may also undergo microbial transformation to yield chemicals with yet other functions. For example, the monoterpene carvone, produced by spearmint (*Mentha spicata*) and caraway (*Carum carvi* L.), inhibits insect herbivory, but also has allelopathic effects when converted by soil bacteria to the chemical, bottrospicatol, which inhibits seed germination of other plants (*19*). Plants may also produce specific terpenes in response to herbivory that are released to attract insect carnivores of the pest that is feeding on the plant, which differ from those released during mechanical damage (*24*). These few examples highlight the complexity of interactions involving terpenes and their transformation products. From an evolutionary perspective, many of these effects seem to be fortuitous, but nonetheless confer distinct advantages that may be important in natural selection (*25*). The possibility that terpenes might induce microbial enzymes that degrade phytotoxic soil contaminants is an intriguing idea that has not been examined.

However, differences in plant tolerance to phytotoxic soils might be related to their ability to induce microorganisms to detoxify the soil environment.

Biodegradation of monoterpenes has recently been reviewed (*7*). As shown in Figure 1, parent structures of monoterpenes include compounds that fall into three groups: acyclic, monocyclic, and bicyclic. The bicyclic compounds have fused rings with C6-C5, C6-C4, and C6-C3 ring systems, each of which pose different problems for degradation. The model monoterpene, is the C6-C5 bicyclic compound, camphor, which serves as a growth substrate for *Mycobacterium rhodochrous* and various strains of *Pseudomonas putida*. The genes for oxidation of camphor by *P. putida* occur on a transmissible plasmid (*26*), which presumably would enhance their ability to move among different microbial populations in the rhizosphere. The best studied camphor degrader is *Pseudomonas putida* G786, which produces the enzyme cytochrome P-450_{CAM}, a monooxygenase that catalyzes hydroxlyation of camphor to 5-exo-hydroxycamphor (*27*). This cytochrome P-450 operates in conjunction with NADH and two other proteins, putidaredoxin and putidaredoxin reductase. During the reaction a monoatomic oxygen is generated that hydoxylates camphor. Of particular relevance to bioremediation, this enzyme is also capable of carrying out reductive dehalogenation, in which electrons are transferred to various organohalides (*28*). This reaction is strongly inhibited by oxygen (*16*), but may still occur at moderate oxygen levels, e.g., 5% or less, which may easily occur in a soil environment where oxygen tensions are typically much lower than in the atmosphere.

A different pathway exists for biodegradation of camphor in *Mycobacterium rhodochrous*. In this bacterium, camphor is hydroxylated at the 6-carbon, afterwhich it follows a similar reaction sequence as for *P. putida* G786. This suggests that there are several different P-450 enzymes capable of catalyzing camphor. Other P-450 enzymes that have been implicated in terpene metabolism include a hydroxylase that catalyses 8-methyl hydroxylation during the first step in degradation of linalool by *P. putida* (*29*). As reviewed by Trudgill (1994), cytochrome P-450 enzymes have also been isolated from bacteria grown on cymene, n-alkanes, and cyclohexane, and are implicated in degradation of the terpenes carene, cineole, and limonene. Despite the intensive studies on camphor and a few other terpenes, the enzymology of terpene degradation is still very limited and degradative systems other than cytochrome P-450s may also be involved. *P. fluorescens* grown on pinene utilizes an epoxide-forming monooxygenase located in the water soluble fraction of cell extracts for the first step in catabolism of this terpene (*30*), and still other pathways are suggested for other *Pseudomonas* strains (*7*). For this reason, terpenes cannot be presumed to induce enzymes that will have broad specificity toward various xenobiotics as observed with cytochrome P-450CAM.

Presently, most research on terpenes has focused on leaves and stems of plants, in which terpenes function to prevent herbivory. However, terpenes are also produced by plant roots, as shown for example with the camphanol glycoside, shionoside C, which has recently been isolated from the roots of *Aster tataricus* (*8*). A major research need is a survey to identify plants that produce high amounts of terpenes in the roots. However, not all terpene producing plants may be beneficial, depending on the target contaminant and the microflora in the rhizosphere. In addition to having positive

Downloaded by PENNSYLVANIA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch018

Downloaded by PENNSYLVANIA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch018

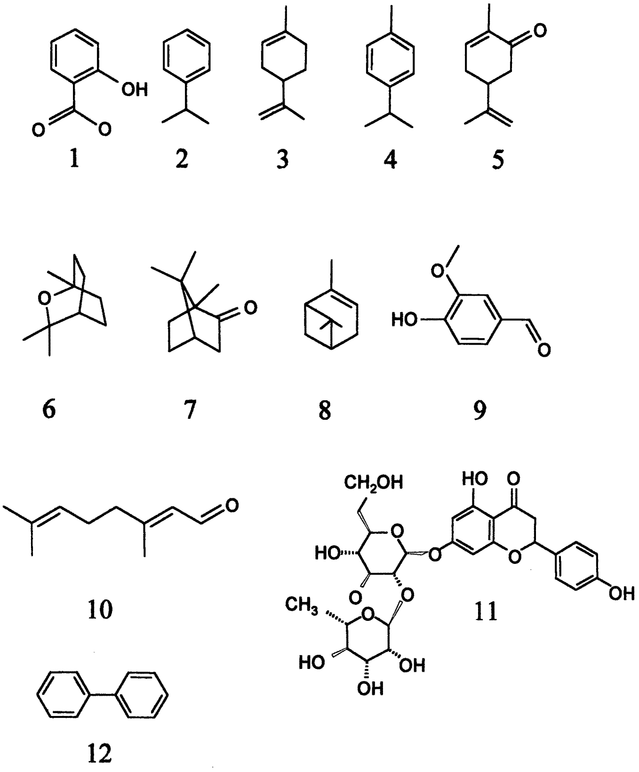

OH
O
O
1
2
3
4
O
5
O
6
O
7
8
O
HO
O
9
O
10
CH2OH
HO O
O
HO
O
O
O
CH3
O
HO
OH
OH
OH
11
12

Figure 1. Representative plant compounds of interest for bioremediation: 1) salicylic acid; 2) cumene; 3) limonene; 4) cymene; 5) carvone; 6) cineole; 7) camphor; 8) pinene; 9) vanillin; 10) citral; 11) naringin; 12) biphenyl.

effects on inducing cometabolism, there has also been a report of an inhibitory effect of certain monoterpenes on methane monooxygenase (*31*). In pure culture studies with various methanotrophs, the inhibition effect was dependent on the degrader stain and monoterpene type. The inhibitory effect of monoterpenes on methane oxidation was greatest with unsaturated, cyclic hydrocarbon forms (e.g., (-)-α-pinene, (S)-(-)-limonene, (R)-(+)-limonene, and γ-terpinene). Lower levels of inhibition occurred with oxide and alcohol derivatives ((R)-(+)-limonene oxide, α-pinene oxide, linalool, α-terpineol) and a noncyclic hydrocarbon (β-myrcene). Isomers of pinene inhibited activity to different extents. These data, which are in contrast to the many reports of positive effects of camphor for inducing cometabolism, suggests that terpenes may have many different effects on bacterial activities in nature. Terpenes are also degraded by fungi, and can be transformed to a variety of different compounds (*32*). This introduces another level of complexity since terpenes produced by plants may be converted to other terpenes with effects differing from those of the parent compound.

Downloaded by PENNSYLVANIA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch018

Salicylic Acid

One of the most versatile and interesting signal compounds in the environment is salicylic acid, which plays a critical role in plant defense against pathogen invasion, but which also has been shown to be an effective inducer for catabolism of a number of xenobiotics. In plant pathology, salicylate has been studied extensively for its role in induced systemic resistance (*33*). This induced systemic response in plants is a generalized multi-array defense that occurs during herbivory or attack by plant pathogens. After induction, salicylate moves through the plant to cause expression of defense genes that help to suppress further attack of the pathogen. Defense genes include the release of phytoalexins, increased production of repellant terpenoids, production of phenolics, and in some cases, interplant signaling. In addition to plant disease tolerance, and herbivory protection, stresses such as ozone and ultraviolet-C radiation (potent active oxygen species generators) also increase endogenous salicylate levels (*34*). In the rhizosphere, bacterial production of salicylate is implicated as an important mechanism for plant protection by plant beneficial disease suppressive bacteria. Salicylate is produced by rhizosphere pseudomonads, and may have siderophore functions under iron stress conditions (*35*).

The precise mechanism and the actual role of salicylic acid in induced systemic resistance are still unclear, as other chemical elicitors are also apparently involved in plant defense responses (*36,37*). As a signal compound, however, salicylate appears to be an integral component of many systems (*38*). In tobacco, several different compounds other than salicylate elicit the systemic acquired resistance response that protects against tobacco mosaic virus, some of which still act via salicylic acid for signal transduction (*36*). In addition to functioning as a signal within the tobacco plant, salicylate is converted to methyl salicylate, a volatile compound that is produced by tobacco plants infected with tobacco mosaic virus (*39*). After absorption by adjacent plants, methyl salicylate is converted back to salicyclic acid, and thereby functions as an airborne signal that activates disease resistance and the expression of defence-related genes in neighbouring plants and in the healthy tissues of the infected plant.

With respect to biodegradation, salicylic acid has been studied for completely different reasons than those described above. Salicylate is an inducer of many different biodegradation enzymes, including those involved in cometabolism of PAH, trinitrotoluene (TNT), and PCBs. One explanation for the central role of salicylate in these different pathways is that the complete pathways for degradation of these xenobiotics probably have evolved in a modular fashion by gene or operon recruitment (*40*). Catabolism of benzene, toluene (and xylenes), naphthalene and biphenyl all occur via catechol and the extradiol (*meta*) cleavage pathways. In various *Pseudomonas* species, the common *meta* cleavage pathway operons, adjacent to the gene for catechol 2,3-dioxygenase, are highly homologous, suggesting they share a common ancestry. As argued by Williams (1994), this common module may have become fused to a gene or genes producing products that can convert chemicals such as benzoate, salicylate, toluene, benzene, and phenol to catechol, thus forming the lower pathway operons found in modern strains. The upper pathway operons might then have been acquired as a third module at a later stage, thus increasing the catabolic versatility of the host strains.

Recently, a salicylate inducible toluene monooxygenase enzyme from *Burkholderia cepacia* (*41*) has been shown to be useful for enhancing the degradation of trichloroethylene by bacteria inoculated into the rhizosphere of wheat (*Triticum aestivum* L.) plants (*42*). A transposon integration vector was used to insert tomA+ into the chromosome of *P. fluorescens* 2-79, producing a stable strain that expressed constitutively the monooxygenase. For comparison, a salicylate-inducible *P. fluorescens* strain that degraded TCE was constructed, and was shown to degrade TCE at comparable rates. In this work, the authors chose the constituitive strain for the rhizosphere studies to eliminate the need for incorporating salicylate into the soil. When inoculated onto wheat, the strain achieved a high level of root colonization, with 4 x 10^6 cfu/cm of root. Rhizoremediation of TCE was demonstrated in which closed microcosms degraded an average of 63% of the initial TCE in 4 days compared to the 9% of the initial TCE removed by negative controls consisting of microcosms containing wild-type *P. fluorescens* 2-79-inoculated wheat, uninoctilated wheat, or sterile soil. Whether this activity might also have been obtained with plants inoculated with the salicylate induced strain was not determined, but is an obvious question for future studies. One concern with inoculation is that the strain may only have temporary survival at effective numbers required for bioremediation. Interestingly, research by other labs has been conducted in which salicylate was applied through irrigation water to maintain plant growth promoting bacteria in the rhizosphere of tomato (*Lycopersicum esculentum*) (*43*).

Flavonoids

Similarly to terpenes, flavonoids are also of interest in both microbial ecology and bioremediation. Flavonoids are compounds with a C6-C3-C6 structure, with the C6 structures consisting of benzene rings. Flavonoids produced by legume roots are

Downloaded by PENNSYLVANIA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch018

signal molecules that act both as chemo-attractants and *nod* gene inducers for the symbiotic *Rhizobium* partner. In studies with alfalfa (*Medicago sativa*), growth of the plant under nitrogen-limiting conditions results in enhancement of expression of the flavonoid biosynthesis genes chalcone synthase and isoflavone reductase and in an increase in root flavonoid and isoflavonoid production, as well increasing *Rhizobium meliloti nod* gene-inducing activity of the root extract (*44*). These results indicate that in alfalfa roots, the production of flavonoids can be influenced by the nitrogen nutrition of the plant. In addition to symbioses with rhizobium, flavonoids are also involved in regulation of mycorrhizal symbioses (*45*), and influence the colonization of grasses by free living nitrogen fixing bacteria (*46*). Using strains marked with the lacZ reporter gene, intercellular colonization of wheat roots by *Azorhizobium caulinodans* and other diazotrophic bacteria has been observed by light and electron microscopy. These bacteria enter the roots of wheat at high frequency at the points of emergence of lateral roots. Biostimulation with the flavonoid naringenin at 10 and 100 mmol m^{-3} significantly stimulated root colonization. In studies with another diazotroph, colonization by *A. brasilense* was also stimulated by naringenin and by other flavonoid molecules.

Root colonizing pseudomonads apparently influence flavonoid production in plant roots, and have been shown to thereby enhance the colonization of roots by *Rhizobia* species that respond to flavonoids as signal compounds for nodule formation (*47*). Co-inoculation with fluorescent *Pseudomonas* and the spore-forming *Bacillus* strains, which are predominant microorganisms found in the rhizosphere and rhizoplane of healthy chickpea (*Cicer arietinum* L.) plants, resulted in a significant increase in nodule weight, root and shoot biomass and total plant nitrogen. Ethyl acetate extracts of culture supernatant fluids when applied to seeds also resulted in enhancement of flavonoids in the roots, suggesting that these rhizobacteria may produce chemicals that act as signal molecules for induction of plant flavonoids in plant roots. Among the candidate molecules are fluorescent siderophores and salicylic acid produced by these rhizobacteria.

Degradation of flavonoids by *Rhizobia, Agrobacterium*, and certain pseudomonads has been studied for *nod* gene-inducing flavonoids. In *Rhizobia*, the pathway involves cleavage of the C-ring of the molecule, which yields conserved A- and B-ring products among the metabolites (*48*). In contrast, *Pseudomonas putida* degraded quercetin via an initial fission in its A-ring, and *Agrobacterium tumefaciens* displayed a nonspecific mode of flavonoid degradation that yielded no conserved A- or B-ring products. When incubated with rhizobia, flavonoids with OH substitutions at the 5 and 7 positions yielded phloroglucinol as the conserved A-ring product, and those with a single OH substitution at the 7 position yielded resorcinol. A wider range of structures was found among the B-ring derivatives, including *p*-coumaric, *p*-hydroxybenzoic, protocatechuic, phenylacetic, and caffeic acids. Other experiments on flavonoid degradation have been carried out with the fungus, *Botrytis cinerea*, and the flavonoid 6-prenylnaringenin (*49*). Preincubation was required to induce the enzymes necessary for degradation, which was dependent on molecular oxygen and NADPH. Low amounts of FAD also were necessary for maximal enzyme activity. The enzymatic activity was not inhibited by various inhibitors of cytochrome P-450 tested,

Downloaded by PENNSYLVANIA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch018

which suggested that this enzyme does not belong to the monooxygenases dependent on cytochrome P-450, but to those dependent on FAD.

PAH Degradation in the Rhizosphere

Plants have been shown to have practical application for cleanup of polycyclic aromatic hydrocarbons (*50*), which appears to involve a general rhizosphere effect that is not well understood. In a recent experiment comparing PAH degradation, four plant species were grown in a previously landfarmed soil and a noncontaminated control soil spiked with selected PAHs. After 24 weeks of plant growth, vegetated soils had significantly lower concentrations of the PAHs than unvegetated soils, with 30 to 44% more degradation occurring in the vegetated soils (*51*). Leaching, plant uptake, abiotic degradation, and irreversible sorption were insignificant, suggesting that the primary effect was rhizosphere enhanced biodegradation. Which microorganisms carried out PAH degradation, the pathways utilized, and whether any rhizosphere chemical components were involved in induction of the PAH degradation enzymes, all remain unknown.

There are a number of scenarios by which rhizosphere microorganisms might degrade PAHs. The cytochrome P-450$_{CAM}$ enzyme produced by *Steptomyces griseus* hydroxylates naphthalene to 1 napthol (*52*). Another cytochrome P-450 produced by *Saccharomyces cerevisiae* hydroxylates benzopyrene at several positions to produce benzopyrene 7.8-dihydrodiol, 9-hydroxybenzopyrene, and 3-hydroxybenzopyrene (*10*). Certain bacterial species also are capable of direct growth on individual PAHs as carbon and energy sources (*53*). In experiments with bacteria capable of growth on naphthalene, fluorene, phenanthrene, anthracene, fluoranthene and pyrene, all of the individual strains were able to cometabolize other PAHs, but were also subject to toxicities of certain compounds. Naphthalene was toxic to all strains not isolated on this compound. Interesting, when the strains were mixed together, these toxicity effects, including that with naphthalene, were allieviated.

Plant Induction of PCB Cometabolism

PCB degradation in the rhizosphere can potentially be achieved by a number of biphenyl degrading bacteria that are broadly distributed in all soils. Suspected natural substrates for these bacteria are benzene, naphthalene and other aromatic hydrocarbons (*54*). Plant products that support the growth of PCB degraders include flavonoids and terpenes (*55-57*). To date however, no studies have actually shown enhanced degradation of PCBs in the plant rhizosphere. In one study, several rhizobial strains catabolized polychlorinated biphenyls (PCBs), and contain genes that hybridize to the *bphABC* genes from the PCB degrader *Comamonas testosteroni* strain B-356 (*58*). However, no experiments were conducted that show these bacteria actually carry out PCB degradation in the rhizosphere. Another important study

Downloaded by PENNSYLVANIA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch018

Downloaded by PENNSYLVANIA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch018

showed that a genetically modified, PCB degrading pseudomonad is competitive in soil and induces the *bph* genes in the rhizosphere (*59*). Although this work represents a possible breakthrough for using plants to degrade PCBs, there is still a paucity of research on this topic.

One problem is that there is a great deal of variation in the ability of different strains to metabolize PCBs, which is reflected in different congener use patterns. In soil surveys, it has been shown that some strains that hybridize with *bph* gene probes for PCB degradation, use only a few PCBs, whereas only a few are highly effective (*54,60*); thus the interest in genetically modified bacteria and bioaugmentation. Whether the rhizosphere can provide a conducive environment for PCB degrading bacteria depends on which bacteria are selected by growth on root derived carbon, the production of inducing substrates for cometabolism of PCBs, and the competitiveness of PCB degraders in using these carbon sources as compared to other bacteria. In the experiment by Brazil and coworkers (*59*), rhizosphere competence of the *bph*-transposon modified pseudomonad was assessed in colonization experiments in nonsterile soil microcosms on sugar beet roots. The modified strain was able to colonize as efficiently as a marked wild-type strain, and *in situ* expression of the *bph* genes was observed when the bacterium was inoculated onto sugar beet seeds. These experiments demonstrated that rhizosphere-adapted microbes can be genetically manipulated to metabolize novel compounds without affecting their ecological competence. Further work needs to be conducted to determine whether bioaugmentation would work under nonsterile conditions.

An important consideration with indigenous bacteria is that degradation is often carried out by consortia rather than individual strains. Effective PCB degradation is dependent on the removal of inhibitory metabolites generated from chlorobenzoates in the lower pathway. Hernandez and coworkers have suggested that there are at least three types of bacteria involved in the aerobic mineralization of PCBs (*61*). In a study examining catabolic features that are lacking in biphenyl-degraders, selected bacteria were tested for their ability to utilize chlorinated acids and to cometabolize Aroclor 1254 and dibenzop-dioxane (dioxin). A broad and variable substrate specificity of the biphenyl dioxygenase among strains was noted by the range of cometabolism of total PCB congeners and dioxin. Growth on chloroalkanoic acids, measured with 2-chloropropionate, occurred for 87% of the strains. In contrast, 72, 66, and 28% of the strains could use 3-chloropropionate, 4-chlorobutyrate, and trans-3-chlorocrotonate, respectively. Only one strain, *Pseudomonas fluorescens* K3, could utilize chloroacetate. None of the biphenyl-utilizers grew on 2- or 4-chlorobenzoate, and only five strains grew on 3-chlorobenzoate. In examining the distribution of these bacteria in three other soils, acetate and benzoate-utilizers were enumerated at 10^6 cfu g^{-1}, whereas chloroacetate- or chlorobenzoate-utilizers were not detected. Thus the inability of biphenyl-degraders to utilize chloroacetate, a central intermediate in the *meta* fission pathway, may be relevant to the incomplete catabolism of PCBs by many biphenyl-utilizers (*61*).

Plant beneficial effects for enhancing the biodegradation of chlorobenzoates have been shown for 3- and 4-chlorobenzoate, and involves growth-linked metabolism stimulated by organic acids and sugars contained in root exudates (*6*). Other more recalcitrant chlorobenzoates may require the introduction of bacterial inoculants or genes. In experiments with 2,5-dichlorobenzoate, introduction of a degrader strain into soils with plants resulted in greatly enhanced degradation rates, as well as increased survival of the degrader strain in the plant rhizosphere (*62*). This strain, which carried the genes for this substrate on a plasmid, also apparently conjugated with other rhizosphere bacteria that could be isolated after soil inoculation. After the substrate was catabolized, the plasmid was eventually lost from the majority of the population, but was maintained at a higher level in rhizosphere isolates than in bacteria reisolated from the bulk soil.

Terpene Enhanced PCB Degradation

PCBs were first shown to undergo cometabolism by bacterial strains grown on biphenyl, which is the non-chlorinated analog of these compounds. This substrate induces the biphenyl dioxygenase genes that carry out ring fission to yield chlorobenzoates. The observation that this inducing substrate is relatively rare in nature, whereas biphenyl degrading bacteria are ubiquitous led to the idea that other naturally occurring compounds may serve as the natural substrates for *bph* genes. This was investigated by Hernandez *et al.* (*57*) and by Gilbert and Crowley (*56*), who showed that a variety of terpenes could induce PCB degradation. Terpene enrichment of soils using natural substrates including orange peels and pine needles resulted in an increase of 5 orders of magnitude in biphenyl-degrading bacteria (*57*). Further studies showed that selected gram-positive bacteria grown on cymene and limonene metabolized 80% of Aroclor 1242 during a 6-day period. In the studies by Gilbert and Crowley, comparisons of terpenes used to induce PCB cometabolism by *Arthrobacter* sp. strain B1B showed that cymene was highly effective for causing the degradation of PCBs, as were carvoen and several other monoterpenes (*56*). Rates of degradation induced by terpenes showed that the most effective monoterpenes were more effective inducers of PCB cometabolism than were biphenyl, but also suggested that some terpenes such as carvone are toxic to bacteria at high concentrations. Detailed studies of the kinetics of PCB degradation by *Arthrobacter* B1B showed that cells were maximally induced by carvone at 5 µM (*63*). This is important since even low levels of monoterpenes diffusing in a vapor phase may induce cometabolism at extremely low concentrations of the inducing substrate.

In further research aimed at characterizing the induction of PCB degradation by various strains, terpenes only caused induction of gram-positive bacteria including *Rhodococcus erythropolis*, but did not induce two gram-negative organisms including the well-studied *Pseudomonas putida* LB400 and *Ralstonia eutrophus* H850 (*63*). Hernandez *et al.* also reported that none of the bacteria isolated after terpene enrichment of biphenyl degraders had genes that hybridized to a *bphA* gene probe that targets gram-negative isolates, although more recent work with another probe for the *bphC* gene showed that *Ralstonia eutrophus* was induced on carvone (*64*). This suggests that there are a variety of *bph* genes that are differentially induced by different compounds. Investigation of other natural substrates that might induce gram-

Downloaded by PENNSYLVANIA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch018

negative bacteria has since revealed that the compound salicylic acid is effective for upregulating the expression of PCB degradation in *Ralstonia eutrophus* H850 (*65*). This is not surprising, since most enzymes that function in biphenyl degrading bacteria function both in the naphthalene and biphenyl degradative pathways (*66*). As discussed earlier, salicylate is an effective inducer of the *nah* genes.

Enrichment of Xenobiotic Degraders on Terpenes

A key question in the biochemical ecology of terpenes is the determination of bacterial species that can degrade these compounds, and whether these same species are also capable of effective biodegradation of xenobiotics. In studies conducted in our laboratory, we have enriched soil with terpenes to select out isolates of biphenyl degrading bacteria. However, none of the isolates enriched on selected terpenes were effective for degradation of PCBs, even though all were capable of growth on biphenyl. To further examine the effect of terpenes on microbial community structure and the number of different terpene degrading bacterial populations in soil, we have conducted soil enrichments with selected terpenes, biphenyl, salicylate and flavonoids. Four of the monocyclic terpenes chosen are highly similar chemical analogs of salicylate. In these previously unpublished studies, the soils were amended with 100 ppm of each compound and allowed to incubate for 10 days. Community structures were analyzed by denaturing gradient gel electrophoresis (DGGE) of 16S rDNA amplified from bulk soil DNA. To differentiate between nongrowing and growing populations, the soils were supplemented with a brominated DNA analog, bromodeoxyuridine (BrdU) 24 hours prior to DNA extraction. Following DGGE of the unlabled and BrdU-labeled DNA, the gels were subjected to image analysis.

Results of this study showed that many different bacteria were enriched on the various plant substrates, but that similar communities were generated on a few different groups of compounds (Figure 2). To further analyze the community similarities, the data were subjected to peak fitting to quantify 16S rDNA band associated with each group. These data were then used for correspondence analysis to plot similarities with an ordination diagram (Figure 3). Interestingly, the resulting communities could not be grouped based on structural similarities in the substrates. Communities enriched on carvone, cumene, and camphor were nearly identical to each other. Another cluster occurred with cymene, biphenyl, and citral. The most dissimilar communities were generated on pinene and naringin. This community-based approach may be useful for screening different plant chemicals that may be effective inducers of xenobiotic degraders for a variety of xenobiotic substances.

Summary

Among the different phytochemicals that are produced by plants, some of the most interesting compounds are those that serve as signal compounds for plants and

Downloaded by PENNSYLVANIA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch018

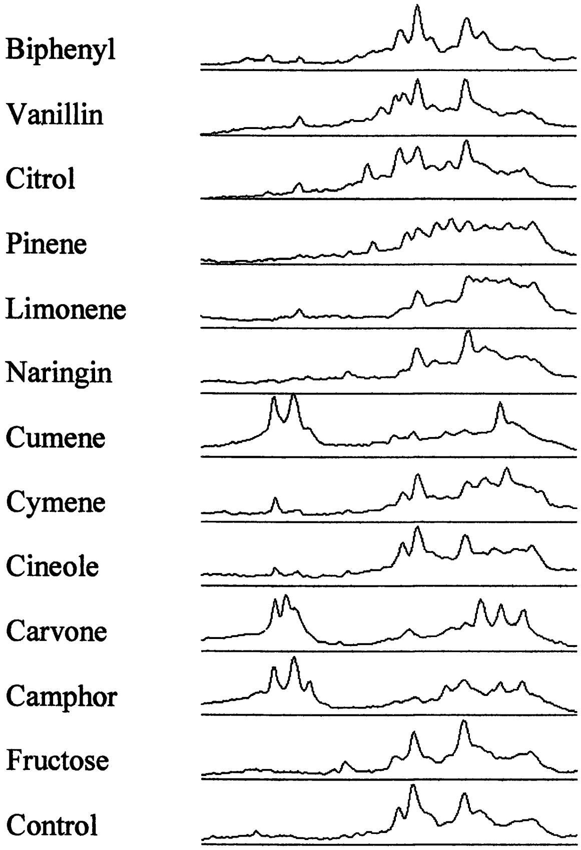


Figure 2. Community analysis of soil bacteria enriched by selected phytochemicals (100 ppm). Line profiles generated by image analysis of BrdU-labeled 16S rDNA separated by denaturing gradient gel electrophoresis (DGGE).

Downloaded by PENNSYLVANIA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch018

Downloaded by PENNSYLVANIA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch018

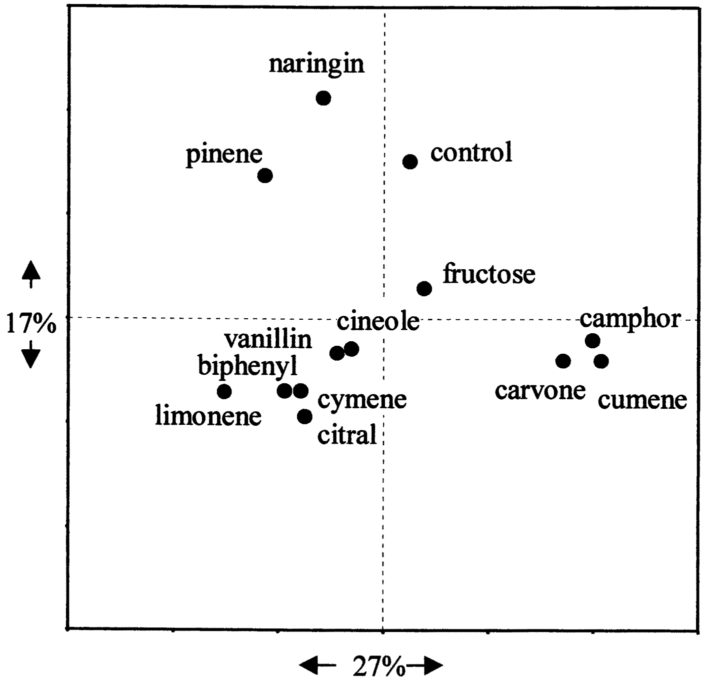


Figure 3. Correspondence analysis of bacterial communities showing relative similarities and differences in community structures after enrichment on selected phytochemical inducers of PCB cometabolism. Ordination plots were generated from peak analysis of DGGE gel for BrdU-labeled 16S rDNA.

microorganisms. These compounds have tremendous versatility and serve many ecological functions, including the possible enhancement of xenobiotic degradation. Salicylic acid functions in the inducibile systemic acquired resistance response in plants, serves as a siderophore for plant beneficial microorganisms, and functions as an inducer of the napthalene, toluene, and biphenyl degradation genes in various bacteria. Flavonoids serve as selective growth substrates for PCB degraders and may also induce genes for their degradation that may function for degradation of structurally related xenobiotics. Terpenes function in plant signaling to other plants, to insects, and to bacteria. As with salicylic acid, certain terpenes are also highly effective for inducing degradative pathways that cometabolize PAHs, PCBs, aromatic hydrocarbons and other substances. Interestingly, there also appear to be interactions between salicylic acid and terpenes, in which salicylate may be involved in inducing terpene production in plants. In this respect, there is a fascinating convergence of research on the biochemical ecology of a few selected chemicals that are now being studied by plant pathologists, entomologists, and microbiologists. A central feature of these substances is that they are all small, mobile chemicals that are capable of being taken up by cells, and interacting through signal transduction pathways to induce production of specific enzymes.

Currently, much of the research on cometabolic degradation of xenobiotics in soils is on individual microbial strains, and the isolation or genetic modification of bacteria for bioaugmentation. However, the greater challenge is with microbial consortia and methods for their manipulation using plants. There are also thousands of different chemicals that might be screened for their effects on soil communities. New methods for rapid analysis of communities and the expression of genes in the environment will likely result in a breakthrough in this research field. In particular, DNA microarrays are now being designed for microbial community analysis, and eventually DNA chips may be designed to screen for the presence and expression of biodegradation genes. Many of the pathways for biodegradation of xenobiotics appear to have common enzymes, which probably reflects their recent evolution by operon assembly into pathways, and by gene recruitment. As shown for PCBs however, there is also a great deal of variation in the versatility of different enzymes for various substrates. It will remain difficult in this respect to determine from gene probe methods exactly how effective a soil community will be at degrading a particular substance. Thus, there is a considerable opportunity for deliberate construction of new microorganisms and plants or for the use of specifically designed plant-microbe systems for cleanup of environmental contaminants.

References

1. Wrigley, S. *Phytochemical Diversity: a Source of New Industrial Products.* Royal Soc. of Chemistry: Cambridge, 1997.
2. Nichols, T. D.; Wolf, D. C.; Rogers, H. B.; Beyrouty, C. A.; Reynolds, C. M. *Water Air Soil Poll.* **1997**, *95,* 165-178.

Downloaded by PENNSYLVANIA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch018

3. Jordahl, J. L.; Foster, L.; Schnoor, J. L.; Alvarez, P. J. *J. Environ. Toxicol. Chem.* **1997**, *16*, 1318-1321.
4. Crowley, D. E.; Alvey, S.; Gilbert, E. S. In *Phytoremediation of Soil and Water Contaminants*, Kruger, E. L., Anderson, T. A. and Coats, J. R., Eds. Am. Chem. Soc.: Wash. DC., 1997, pp 20-38.
5. Alexander, M. *Bioremediation and Biodegradation*, Academic Press, New York, 1997.
6. Haby, P. A.; Crowley, D. E. J. Environ. Qual. **1996**, *25*, 304-310.
7. Trudgill, P. W. In *Biogeochemistry of Microbial Degradation*. Ratledge, C., Ed. Kluwer Acad. Pub., Netherlands, 1994, pp 33-61.
8. Cheng, D.; Shao, Y. *Phytochemistry* **1994**, *35*, 173-176.
9. Adriaens, P.; Vogel, T. M. In *Ecological and Applied Microbiology: Microbial transformation and Degradation of Toxic Organic Compounds*.Young, L. Y. and Cernigilia, C. E., Eds. Wiley-Liss: N. Y., 1995, pp 435-486.
10. Wiseman, A.; Woods, L. F. J. J. *Chem. Technol. Biotechnol.* **1979**, *29*, 320-324.
11. Kellner, D. G.; Maves, S. A.; Sligar, S. G. *Curr. Opions. Biotechnol.* **1997**, *8*, 274-278.
12. Heald, S. C.; Jenkins, R. O. *Appl. Microbiol. Biotech.* **1996**, *45*, 56-62.
13. DeWeerd, K. A.; Suflita, J. M. *Appl. Environ. Microbiol.* **1990**, *56*, 2999-3005.
14. Potrawfke, T.; Loehnert, T. H.; Timmis, K. N.; Wittich, R. M. *Appl. Microbiol. Biotechnol.* **1998**, *50*, 440-446.
15. Ely, R. L.; Williamson, K. J.; Hyman, M. R.; Arp, D. J. *Biotechnol. Bioeng.* **1997**, *54*, 520-534.
16. Logan, M. S. P.; Newman, L. M.; Schanke, C. A.; Wackett, L. P. *Biodegradation* **1993**, *4*, 39-50.
17. Stomp, A. M.; Han, K. H.; Wilbert, S.; Gordon, M. P. *In Vitro Cell. Develop. Biol. Plant* **1993**, *29*, 227-232.
18. Connolly, J. D.; Hill, R. A. *Dictionary of Terpenoids*; Chapman and Hall: London, 1991.
19. Harborne, J. B.; Tomas-Barberan, F. A. *Ecological Chemistry and Biochemistry of Plant Terpenoids*: Oxford, 1991.
20. Olmedo, E. C. *Crit. Rev. Microbiol.* **1994**, *20*, 151-160.
21. Gershenzon, J.; Croteau, R. B. In *Lipid Metabolism in Plants*, Moore, T. S. J., Ed.; CRC Press: Boca Raton, FL, 1993, pp 339-388.
22. McCaskill, D.; Croteau, R. *Tetrahedron. Lett.* **1999**, *40*, 653-656.
23. Yani, A.; Pauly, G.; Faye, M.; Salin, F.; Gleizes, M. *Plant Cell Environ.* **1993**, *16*, 975-981.
24. Dicke, M. J. *Plant Physiol.* **1994**, *143*, 465-472.
25. Linhart, Y. B.; Thompson, J. D. *Evol. Ecol. Res.* **1999**, *1*, 151-171.
26. Rheinwald, J. G.; Chakrabarty, A. M.; Gunsalus, I. C. *Proc. Nat. Acad. Sci.* **1973**, *70*, 885-889.
27. Bradshaw, W. H.; Conrad, H. E.; Corey, E. J.; Gunsalus, I. C.; Lednicer, D. *J. Am. Chem. Soc.* **1959**, *81*, 5507.
28. Castro, C. E.; Wade, R. S.; Belser, N. O. *Biochemistry* **1985**, *24*, 204-210.

Downloaded by PENNSYLVANIA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch018

29. Ullah, A. J. H.; Murray, R. I.; Bhattacharyya, P. K.; Wagner, G. C.; Gunsalus, J. C. *J. Biol. Chem.* **1990**, *265*, 1345-1351.
30. Best, D. J.; Floyd, N. C.; Magalhaes, A.; Burfield, A.; Rhodes, P. M. *Biocatalysis* **1987**, *1*, 147-159.
31. Amaral, J. A.; Ekins, A.; Richards, S. R.; Knowles, R. *Appl. Environ. Microbiol.* **1998**, *64*, 520-525.
32. Busmann, D.; Berger, R. G. *Z. Naturforschung Biosci.* **1994**, 49, 545-552.
33. Lin, Z.-P.; Hu, Y.-L. Acta Botan. Sin. **1997**, *39*, 185-188.
34. Enyedi, A. J. *J. Plant Physiol.* **1999**, *154*, 106-112.
35. Maurhofer, M.; Reimmann, C.; Schmidli-Sacherer, P.; Heeb, S.; Haas, D.; Defago, G. *Phytopathology.* **1998**, *88*, 678-684.
36. Malamy, J.; Sanchez-Casas, P.; Henning, J.; Guo, A.; Klessig, D. F. *Molec. Plant Microbe Inter.* **1996**, *9*, 474-482.
37. Van Wees, S. C. M.; Pieterse, C. M. J.; Trijssenaar, A.; Van 't Westende, Y. A. M.; Hartog, F.; Van Loon, L. C. *Molec. Plant-Microbe Inter.* **1997**, *10*, 716-724.
38. Mauch-Mani, B.; Metraux, J.-P. *Ann. Bot.* **1998**, *82*, 535-540.
39. Shulaev, V.; Silverman, P.; Raskin, I. *Nature* **1997**, *385*, 718-721.
40. Williams, P. A.; Sayers, J. R. *Biodegradation.* **1994**, *5*, 195-217.
41. Shields, M. S.; Reagin, M. J.; Gerger, R. R.; Campbell, R.; Somerville, R. *Appl. Environ. Microbiol.* **1995**, *61*, 1352-1356.
42. Yee, D. C.; Maynard, J. A.; Wood, T. K. *Appl. Environ. Microbiol.* **1998**, *64*, 112-118.
43. Colbert, S. F.; Schroth, M. N.; Weinhold, A. R.; Hendson, M. *Appl. Environ. Microbiol.* **1993**, *59*, 2064-2070.
44. Coronado, C.; Zuanazzi, J. A. S.; Sallaud, C.; Quirion, J. C.; Esnault, R.; Husson, H. P.; Kondorosi, A.; Ratet, P. *Plant Physiol.* **1995**, *108*, 533-542.
45. Stafford, H. A. *Bot. Rev.* **1997**, *63*, 27-39.
46. Webster, G.; Jain, V.; Davey, M. R.; Gough, C.; Vasse, J.; Denarie, J.; Cocking, E. C. *Plant Cell Environ.* **1998**, *21*, 373-383.
47. Parmar, N.; Dadarwal, K. R. *J. Appl. Microbiol.* **1999**, *86*, 36-44.
48. Rao, J. R.; Cooper, J. E. *J. Bact.* **1994**, *176*, 5409-5413.
49. Tanaka, M.; Tahara, S. *Phytochemistry.* **1997**, *46*, 433-439.
50. Schwab, A. P.; Banks, M. K.; Arunachalam, M. In *Bioremediation of Recalictrant Organics*, Hinchee, R. E., Anderson, D. B. and Hoeppel, R. E., Ed.; Battelle Memorial Institute: Columbus, OH, 1995, pp 23-29.
51. Reilley, K. A.; Banks, M. K.; Schwab, A. P. *J. Environ. Qual.* **1996**, *25*, 212-219.
52. Trower, M. K.; Sariaslani, F. S.; Kitson, F. G. *Biochem. Biophys. Res. Comm.* **1988**, *157*, 1417-1422.
53. Bouchez, M.; Blanchet, D.; Vandecasteele, J. P. *Appl. Microbiol. Biotech.* **1995**, *43*, 156-164.
54. Pellizari, V. H.; Bezborodnikov, S.; Quensen, J. F.; Tiedje, J. M. *Appl. Environ. Microbiol.* **1996**, *62*, 2053-2058.
55. Donnelly, P. K.; Hegde, R. S.; Fletcher, J. S. *Chemosphere* **1994**, *28*, 981-988.
56. Gilbert, E. S.; Crowley, D. E. *Appl. Environ. Microbiol.* **1997**, *63*, 1933-1938.

Downloaded by PENNSYLVANIA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch018

57. Hernandez, B. S.; Koh, S. C.; Chial, M.; Focht, D. D. *Biodegradation* **1997**, *8*, 153-158.

58. Damaj, M.; Ahmad, D. *Biochem. Biophys. Res. Comm.* **1996**, *218*, 908-915.

59. Brazil, G. M.; Kenefick, L.; Callanan, M.; Haro, A.; De Lorenzo, V.; Dowling, D. N.; O'Gara, F. *Appl. Environ. Microbiol.* **1995**, *61*, 1946-1952.

60. Bedard, D. L.; Unterman, R.; Bopp, L. H.; Brennan, M. J.; Haberl, M. L.; Johnson, C. *Appl. Environ. Microbiol.* **1986**, *51*, 761-768.

61. Hernandez, B. S.; Arensdorf, J. J.; Focht, D. D. *Biodegradation* **1995**, *6*, 75-82.

62. Crowley, D. E.; Brennerova, M. V.; Irwin, C.; Brenner, V.; Focht, D. D. *FEMS Microbiol. Ecol.* **1996**, *20*, 79-89.

63. Gilbert, E. S.; Crowley, D. E. *Abstracts of the General Meeting of the American Society for Microbiology* **1997**, *97*,478.

64. Koh, S. Seoul Maritime University, Seoul, Korea , unpublished data.

65. Singer, A. C.; Gilbert, E. S.; Luepromchai, E.; Crowley, D. E. University. California, Riverside unpublished data.

66. Kuhm, A. E.; Stoz, A.; Knackmuss, H. J. *Biodegradation* **1991**, *2*, 115-120.

Downloaded by PENNSYLVANIA STATE UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch018

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch019

Chapter 19

Designing Herbicide Tolerance Based on Metabolic Alteration: the Challenges and the Future

Dale L. Shaner and Berhane Tecle

Agricultural Research Division, American Cyanamid Company, Princeton, NJ 08543

The major basis for the tolerance of crops to most herbicides is differential rates and routes of herbicide metabolism. In spite of detailed knowledge on these routes of metabolism, it has been very difficult to synthesize new crop selective herbicides by deliberately altering the metabolism rate through rational design. The major problem is trying to predict the substrate affinities for the herbicide detoxifying enzymes that are known to exist in multiple isoforms and that vary greatly among plant species. Furthermore, changing a substituent in a molecule, even at a remote site from the actual site of metabolism, can often alter the pathway of degradation by similar enzymes from different plant species. Thus, a number of analogs with different substituents ("metabolic handles") must be synthesized in order to determine if any of these analogs will be detoxified. An alternative approach to generate herbicide tolerance is to introduce genes for metabolism from microbes into target crops. This approach has been successfully used in a number of crops.

In Pesticide Biotransformation in Plants and Microorganisms; Hall, J., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2000.

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch019

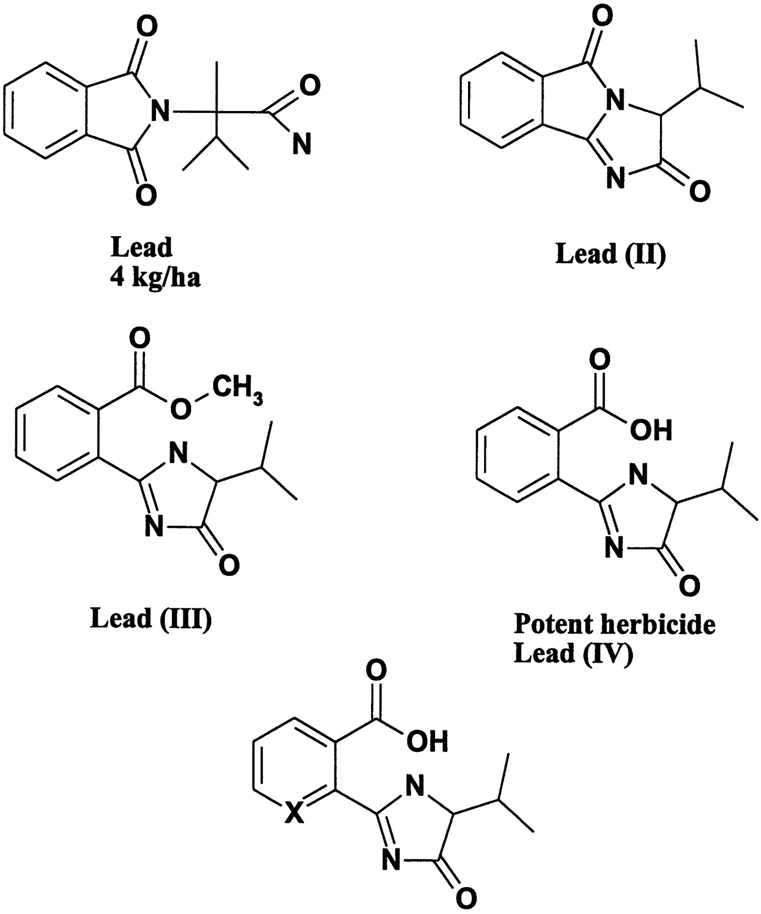
A number of approaches have been taken to discover broad-spectrum herbicides with ideal attributes of efficacy, crop selectivity, environmental and toxicological safety, and low cost. These approaches include the empirical method where a number of compounds are screened on target crops and agronomically important weeds. This approach has been successful in identifying important leads for subsequent structure/activity /selectivity optimization. The other approach that has been explored includes the design of agrochemicals based on biochemical knowledge.

The major basis for the crop selectivity of most of the herbicides on the market is well-documented (*1-4*). These studies clearly show that selectivity of most of these herbicides is due to differential rates and routes of metabolism in the target crops and the weeds. Other mechanisms, such as inherent sensitivity differences at the target site between weeds and crops, are relatively rare, with the exception of the cyclohexanedione and aryloxyphenoxypropionate herbicides that kill grasses through inhibition of acetyl-CoA carboxylase (ACCase). These herbicides do not affect most dicot species due to the insensitivity of ACCase to these herbicides (*5,6*). Relative herbicide tolerance based on differential herbicide uptake and translocation is an even rarer occurrence. In this paper, we will discuss the major challenges in designing crop selective herbicide(s) based only on the knowledge of herbicide detoxification pathways of commercial herbicides in target crops. Examples will be cited in order to illustrate how a small variation in the structure of an optimized molecule results in totally unexpected rates and routes of herbicide metabolism in the target crops, making prediction of herbicide selectivity very difficult. The recent progress in designing herbicide tolerant crops by the biotechnology approach will be also discussed.

Discovery and Structure/Activity/Selectivity Optimization of the Imidazolinone Herbicides

The discovery and development of the imidazolinone herbicides was a result of the empirical approach to herbicide discovery (*7*). The lead compound (**I**) was discovered from random screening that was found to have sufficient activity at 4 kg/ha to warrant the initiation of a synthesis program around this structure (Figure 1). Subsequent structural modification of the lead phthalimide (**I**) led to a number of other promising leads (**II** and **III**) with some interesting biological activities. The best lead (**IV**) was obtained from further structural modifications of (**III**). An analog synthesis program and extensive greenhouse evaluation for 10 years led to the discovery of the site of action of this class of herbicides and the structural requirements for optimum biological activities (*8,9*). The best structural feature, which showed significant activity at the target site, was a

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch019



Lead
4 kg/ha

Lead (II)

Lead (III)

Potent herbicide
Lead (IV)

(V) X=N, CL 243997

Figure 1. Structures of lead imidazolinone herbicides and the optimized analog.

substituted imidazolinone ring with the methyl-isopropyl side chain attached to an aromatic ring with a carboxylic acid function. When the aromatic ring was a pyridine ring, the best herbicidal activity was observed (V). This was again optimized by substitution at the 5-position in the pyridine ring. Various substituents at the 5-pyridine ring showed different selectivity on some crops.

Studies on the mechanisms of selectivity of a number of the promising analogs with substitution at the 5-pyridine ring showed that their crop selectivity is based on the rates and routes of metabolism of these herbicides (*10, 11*). To our surprise, a small variation in the substituent at the 5-position had unpredictable effects on crop selectivity and on the rates and routes of metabolism in different plant species. For instance, the sensitivity of soybean (*Glycine max*), peanut (*Arachis hypogaea*) and maize (*Zea mays*) to the 5-methyl, ethyl, and methoxymethyl substituted imidazolinone herbicides varied (Table I). The pathways of metabolism of these three imidazolinones vary unpredictably in the three crops due to the presence of different metabolic pathways and small differences in the substrate specificity of metabolizing enzymes based on differences at the 5-position.

Table I. Crop Tolerance to 5-Substituted Imidazolinone Analogs (g/ha)

Herbicide substituent	*Soybean*	*Peanut*	*Maize*
5-Methyl	20	200	10
5-Ethyl	500	500	24
5-Methoxymethyl	250	200	6

Peanut appears to have one detoxification pathway of the 5-methyl and ethyl substituted analogs that involve hydroxylation followed by glycosylation (Figure 2). Soybean, on the other hand, has two competing pathways, a hydrophilic pathway similar to that found in peanuts, and a hydrophobic pathway that unexpectedly competes for the 5-methyl, but which can not detoxify this herbicide rapidly enough to allow it to be used selectively on soybeans (Figure 3). Maize also has a hydrophilic pathway, but, unlike peanut and soybean, maize lacks the ability to rapidly conjugate the hydroxylated imidazolinone metabolites to glucose, and thus can not completely detoxify these herbicides. In addition, the selectivity of the mixed function oxidases system in maize differs in that it can rapidly hydroxylate the 5-ethyl substituted analog, but cannot rapidly hydroxylate either the 5-methyl or methoxymethyl analog. Thus, what may appear to be small differences at 5-substituent site in these three imidazolinones

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch019

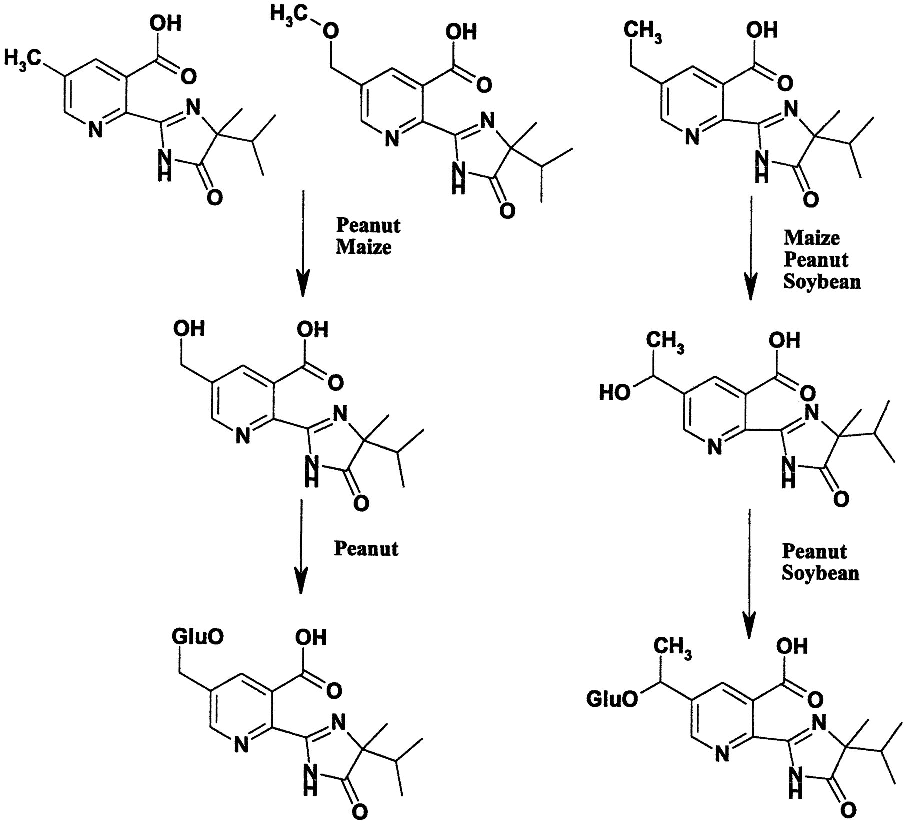

Peanut
Maize
Peanut
Maize
Peanut
Soybean
Peanut
Soybean

Figure 2. Differential routes of metabolism of 5-methy, ethyl and methoxymethyl imidazolinone analogs in maize, peanut and soybean plants.

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch019

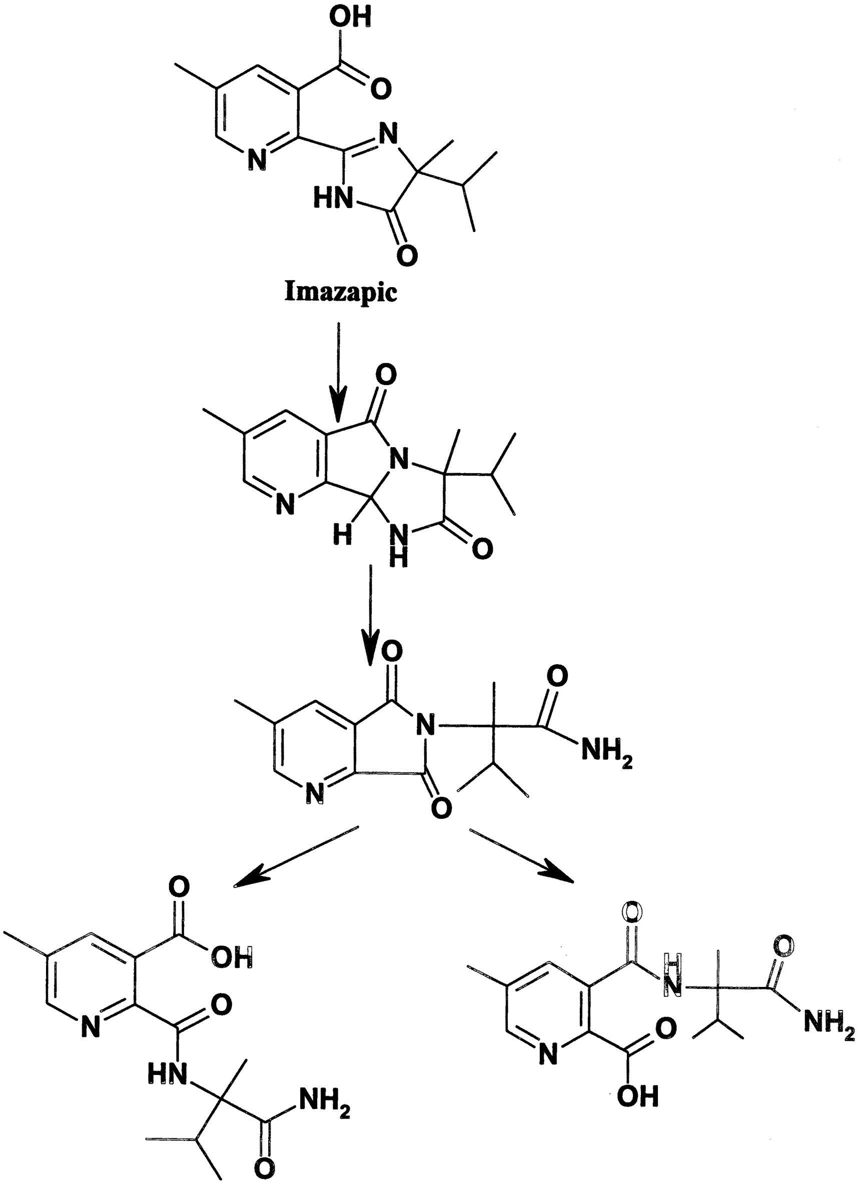

OH
O
N
N
HN
O
Imazapic
O
N
N
H
N
H
O
O
O
N
NH2
N
O
O
OH
N
O
HN
NH2
O
O
H
N
O
NH2
N
O
OH

Figure 3. Metabolic pathway of imazapic in soybean seedlings.

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch019

have large and unpredictable effects on metabolism and, consequently, crop selectivity.

Another example of herbicide tolerance based on differential routes metabolism is imazamethabenz-methyl ((±)-2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1*H*-imidazol-2-yl]-4 (and 5)-methylbenzoic acid) in wheat (*Triticum aestivum*) vs. wild oat (*Avena fatua*) (Figure 4). The tolerance of wheat to imazamethabenz-methyl is a result of hydroxylation of the aromatic methyl groups followed by glycosylation without significant deesterification to the biologically active acid (*12*). In wild oats, imazamethabenz-methyl is bioactivated to the acid, which is systemic, and also a potent inhibitor of acetolactate synthase (ALS), the site of action for this herbicide.

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch019

Metabolism of Sulfonylureas and Other Herbicides

Other studies have also shown the unpredictable metabolic pathways of other herbicides in crops and weeds. For example, DPX-L8747 is a weak inhibitor of ALS, the target enzyme. It undergoes *N*-dealkylation in susceptible grass species and becomes a potent inhibitor of ALS (Figure 5). On the other hand, tolerant plant species such as wheat and soybean detoxify DPX-L8747 via hydroxylation and homoglutathione conjugation, respectively (*3*).

Brown et al, (*13,14*) showed the metabolic fate of other chemically diverse sulfonylurea herbicides is dependent on the functional groups in both the pyrimidine (triazine) ring and aromatic ring (Figure 6). Substituents at the R1 site of sulfonylureas, which is usually an ester, is often attacked by esterases, which detoxify the herbicide. The R2 site on the heterocycle ring of many sulfonylureas is often a site where conjugation occurs while substituents at the R3 site are oxidized as well as the carbon between the R2 and R3 substituted carbons. Hydrolyases can break the sulfonylurea bridge at the sulfonyl side while ureases can break the bridge between the two nitrogens. Which site is metabolized depends on the substituent pattern on the two rings of the sulfonylurea. The most notable example from this class of chemistry is the detoxification of nicosulfuron (2[[[[(4,6-dimethoxy-2-pyrimidinyl) amino] carbonyl]amino]sulfonyl]-*N,N*-dimethyl-3-pyridinecarboxamide) analogs (15, *16*) via hydroxylation of the pyrimidine ring (Figure 7), which is dependent on substituents remote from the site of metabolism (Table II).

Examples from another class of herbicides, which illustrate the substrate specificities among plant species, are the metabolism of chloramben (3-amino-2,5-dichlorobenzoic acid) (*17*). In sensitive species it is metabolized to a glucose ester, which is easily converted back to the active parent compound, whereas in tolerant species, it is detoxified to the stable N-glucoside (Figure 8).

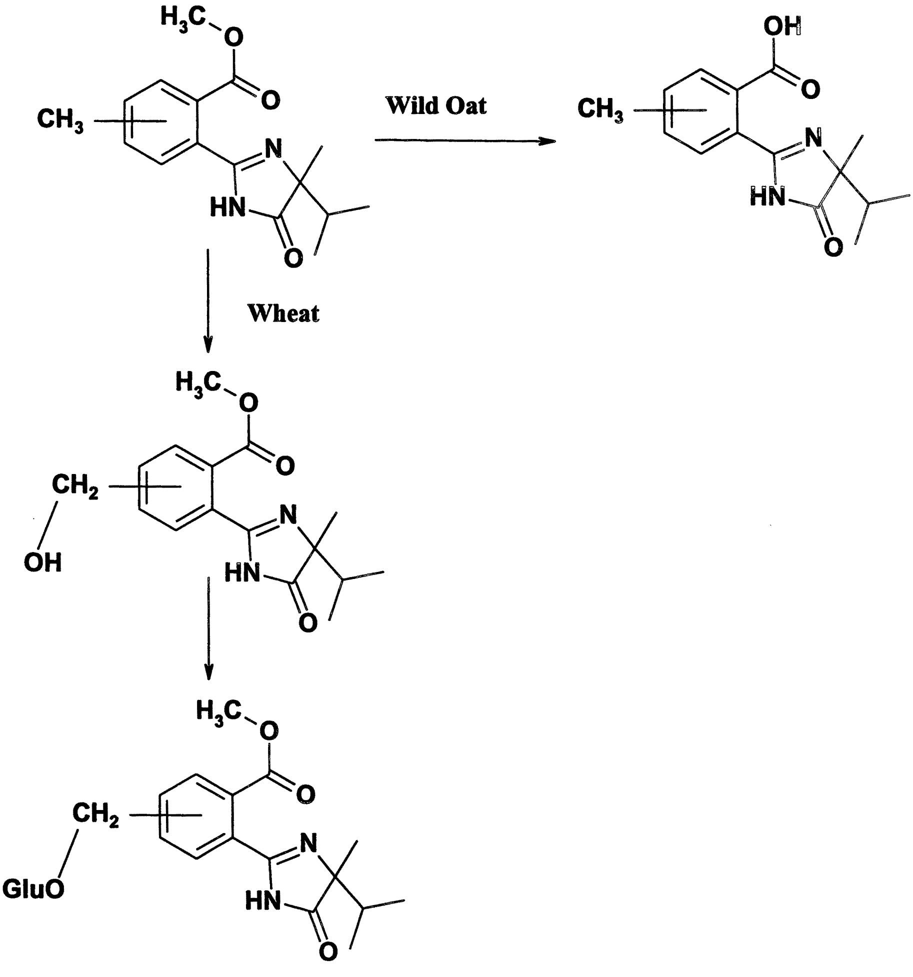


Figure 4. Differential route of metabolism of imazamethabenz in wheat and wild oats.

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch019

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch019

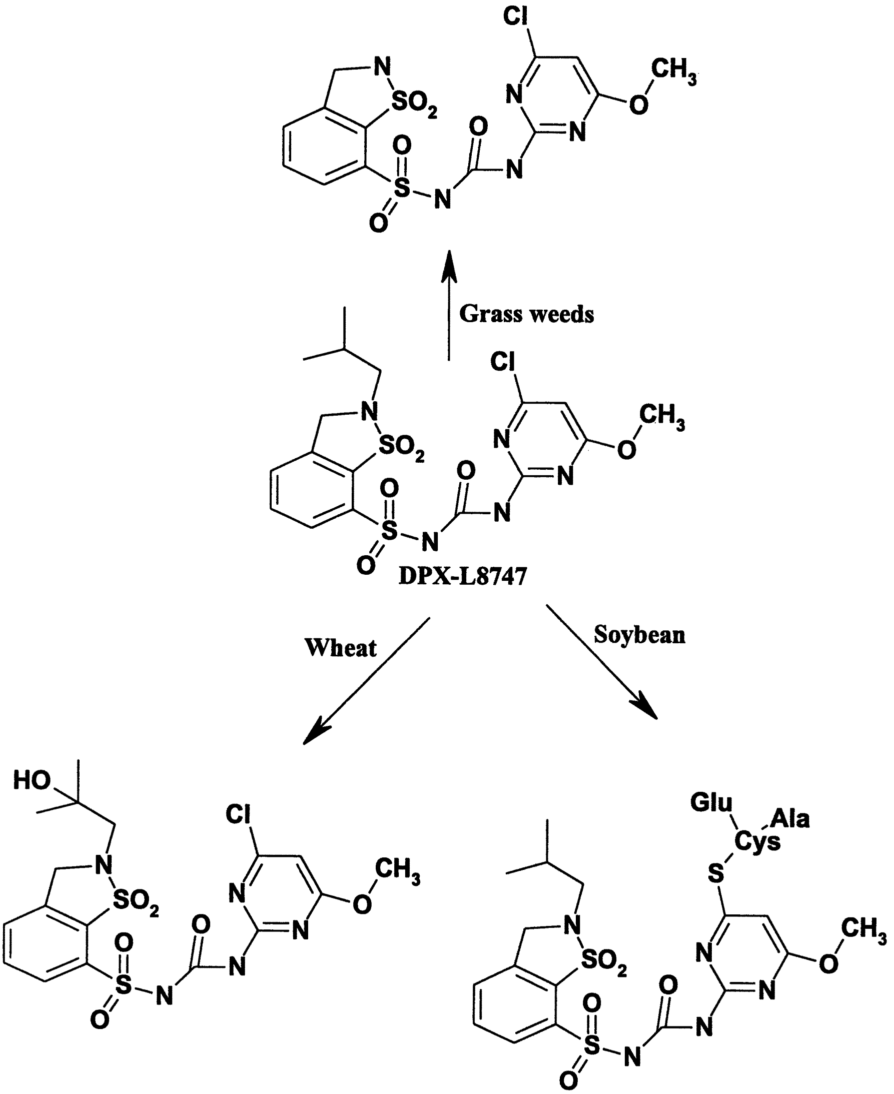


Figure 5. Differential routes of metabolism of DPX-L8747 in crops and grass weeds.

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch019

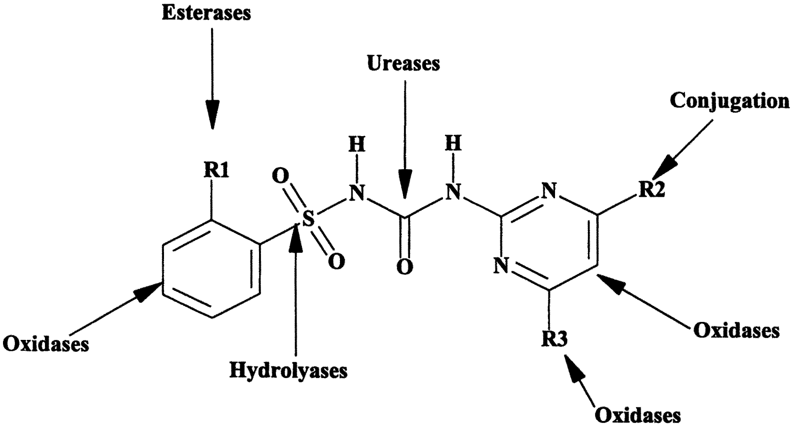

Esterases
Ureases
Conjugation
H
H
R1
O
S
O
N
N
O
N
R2
N
R3
Oxidases
Hydrolyases
Oxidases
Oxidases

Figure 6. Metabolic handles of sulfonylurea herbicides in different plant species.

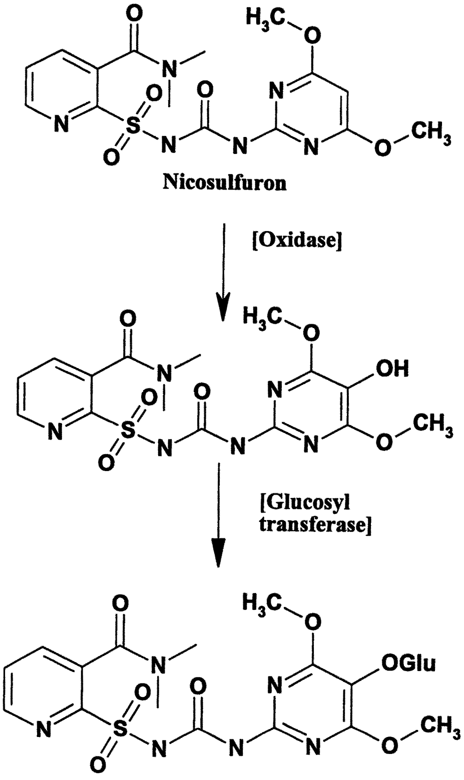

Nicosulfuron
[Oxidase]
OH
[Glucosyl transferase]
OGlu
H3C
O
CH3
N
S

Figure 7. Metabolic pathway of nicosulfuron in maize.

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch019

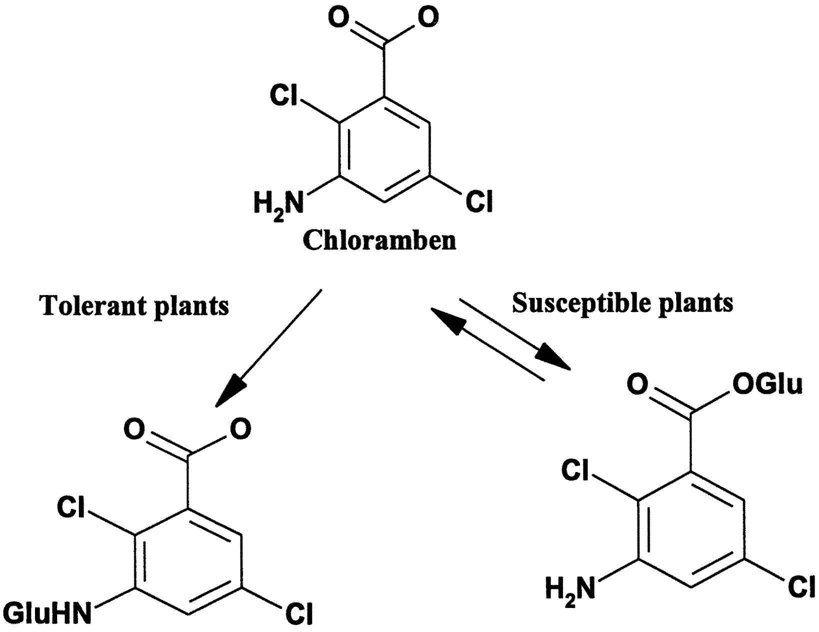
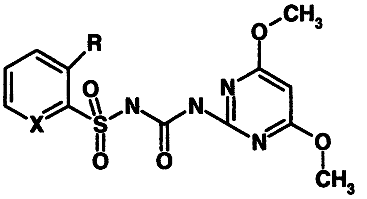

O
O
Cl
H2N
Cl
Chloramben
Tolerant plants
Susceptible plants
O
O
Cl
GluHN
Cl
O
OGlu
Cl
H2N
Cl

Figure 8. Differential routes of metabolism of chloroamben in tolerant and sensitive plants.

Flumetsulam (*N*-(2,6,-difluorophenyl)-5-methyl[1,2,4]triazolo[1,5-α] pyrimidine-2-sulfonamide) in tolerant species such as maize is detoxified via hydroxylation of the aromatic methyl followed by glycosylation (*18*). In sensitive species such as lambsquarters (*Chenopodium album*), it undergoes aromatic hydroxylation at slower rate (Figure 9). This is another example that illustrates the substrate specificity of the hydroxylase enzymes in different plant species.

Table II. Effect of Structural Substituents and Application Rates of Nicosulfuron Analogs on Maize Injury



Structural Moiety		*Maize injury (%)*[a]	
R	*X*	*10 g/ha*	*50 g/ha*
-CON(CH_3)$_2$	-N-	0	0-5
-CON(CH_3)$_2$	-CH-	40-50	60-70
-$COOCH_3$	-N-	95	100
-SO_2CH_3	-N-	80	95

NOTE: [a]injury was measured as visual injury and expressed as percent of control.

Reasons for the Unpredictability of Metabolism of Herbicides

The unpredictability of how different substituents on a herbicide will affect its metabolic fate is a reflection of the complexity of metabolic pathways in plants and of our lack of knowledge. Many of the examples given above involved metabolism via a mixed function oxidase. In most cases these are mediated by P-450 monooxygenases. Monooxygenases in plants and other organisms are highly divergent with very little homology between proteins except the highly conserved region responsible for binding the ferriprotoporphyrin (heme)

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch019

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch019

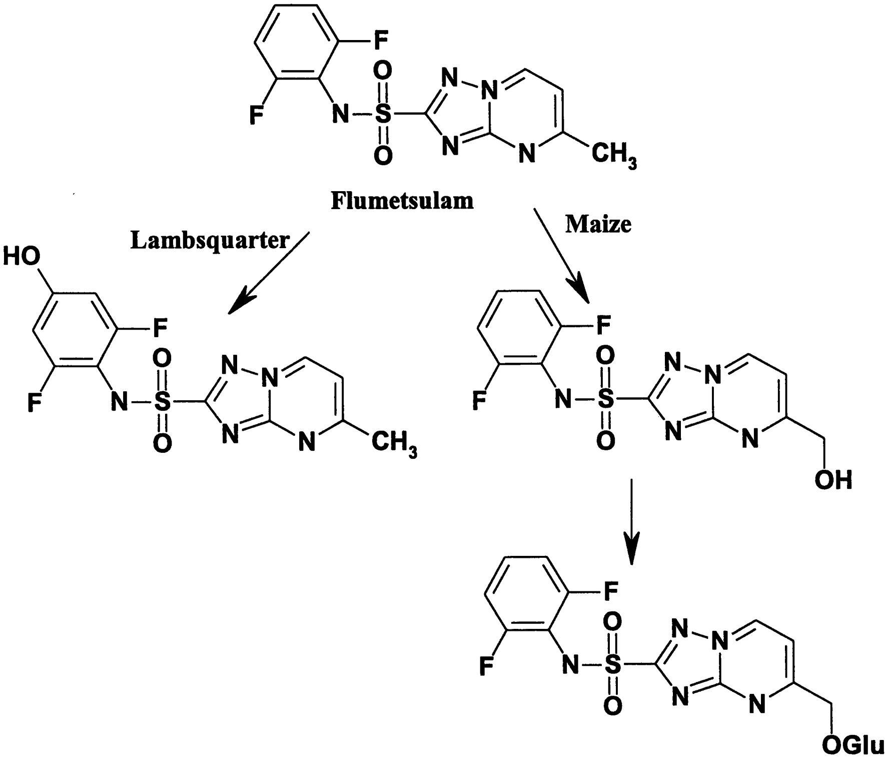

F
O
N-S
O
N-N
N
N
CH3
Flumetsulam
Lambsquarter
Maize
HO
F
F
O
N-S
O
N-N
N
N
CH3
F
F
O
N-S
O
N-N
N
N
OH
F
F
O
N-S
O
N-N
N
N
OGlu

Figure 9. Differential routes of metabolism of flumetsulam.

prosthetic group (*19*). Small changes in enzyme structure outside of this region can greatly alter the catalytic activity of the enzyme. The genes for over 90 plant P-450 monooxygenases representing 26 different gene families have been completed (*19*). Thus, it is not surprising that one cannot predict how changes in chemical structure will affect metabolism given the wide diversity of metabolizing enzymes.

Crop Selectivity through Plant Breeding and Biotechnology

Efforts to incorporate selectivity into herbicides via metabolic handles have been successful for many classes of compounds. However, this has not been the case for all herbicides, particularly for non-selective chemicals such as glyphosate or glufosinate. An alternative approach to modifying the herbicide is to modify the crop. This has been done via classical breeding techniques for certain herbicides such as metribuzin, or through the isolation of genes from microorganisms followed by transfer of those genes into crops. Both approaches have been commercially successful.

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch019

Plant Breeding for Herbicide Tolerance

Metribuzin (4-amino-6-(1,1-dimethylethyl)-3-(methylthio)-1,2,4-triazin-5(4*H*)-one) is an asymmetrical triazine with selectivity in soybeans. However, the selectivity of metribuzin in soybeans can be marginal with significant injury under certain conditions. Work by various researchers (*20-24*) showed that differences in sensitivity among soybean cultivars to metribuzin was due to differences in the rates of metabolism. A highly tolerant soybean variety was derived from Tracy, a popular and widely planted variety at the time (*25*). This new cultivar was released as Tracy M, and it was well accepted. This is one of the few cases where classical breeding techniques were used to increase a crop's tolerance to a herbicide via increased metabolism.

Biotechnology Approaches

2,4-D

2,4-D ((2,4-dichlorophenyl)acetic acid) is a herbicide that controls many broadleaf weeds in monocotyledonous crops such as wheat, barley (*Hordeum vulgare*), and maize. Tolerant crops can rapidly detoxify the herbicide while sensitive broadleaf plants lack this ability. Certain crops, such as cotton (*Gossypium hirsutum*), are extremely sensitive to 2,4-D and can be badly damaged from drift of the herbicide applied to an adjacent crop (*26*). 2,4-D is rapidly metabolized in soil with a half-life of 14 days (*27*). While a number of soil microorganisms are capable of metabolizing 2,4-D, the pathway in *Alcaligenes eutrophus* is the best-characterized (*28*). This microbe can grow in medium in which 2,4-D is the only carbon source (*29*). The pathway of degradation of 2,4-D in this microorganism was determined (*28*) (Figure 10), and it was also found that the genes encoding for most of the enzymes in this pathway were contained in a large plasmid. Although the complete metabolism of 2,4-D requires 6 enzymatic steps (Figure 10), the first breakdown product of 2,4-D in the pathway, 2,4-dichlorophenol, was shown to be 50- to 100-fold less phytotoxic than the parent compound (*26*). This could be enough to confer tolerance to a sensitive plant. The gene encoding for this enzyme (*tfdA*) was isolated and cloned. (*30*), and the gene product was shown to be a ferrous iron dependent dioxygenase (*31*). Since the bacterial gene used GTG as it's start codon, this had to be modified to an ATG to allow it to be correctly recognized and expressed in plants (*28*). The gene was further modified in the region upstream of the ATG so that it would be more easily read by a wide number of plants (*28*).

Two groups. (*32, 33*) successfully transformed Coker 312 cotton with *tfdA* gene constructs and were able to select lines with increased tolerance to 2,4-D, up to 3 times the field use rate. Transgenic tobacco (*Nicotiana spp.*) plants, which carried the *tfdA* construct, metabolized 2,4-D 11- to 85-fold faster than untransformed tobacco (*32*). Presumably the same mechanism was operating in the 2,4-D tolerant cotton. Although 2,4-D tolerant cotton transformants have been provided to public and private breeders in the U.S. and Australia, no commercial lines have been developed and released (*28*).

Bromoxynil

Bromoxynil (3,5-dibromo-4-hydroxybenzonitrile) is used to control broadleaf weeds in a variety of tolerant crops including cereals, and certain varieties of maize and alfalfa (*Medicago sativa*) (*33*). This herbicide is a potent photosystem II inhibitor and is rapidly metabolized in tolerant crops to 3,5-dibromo-4-hydroxybenzamide and then to 3,5-dibromo-4-hydroxybenzoic acid (*34*), both of which are 100-fold less toxic than the parent material (Figure 11). Bromoxynil is also rapidly metabolized by soil microorganisms to 3,5-dibromo-4-hydroxybenzoic acid (*33*). This reaction appears to occur in a single step, bypassing the amide intermediate seen in tolerant crops.

Researchers (*35*) isolated a bacterium, *Klebsiella ozaenae*, from bromoxynil-contaminated soil which could use this herbicide as its sole nitrogen source. This isolate contained a nitrilase with high specificity for bromoxynil, and the gene encoding for the nitrilase was contained on a plasmid (*36*). This gene was isolated, cloned, and placed in a construct used to transform cotton.

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch019

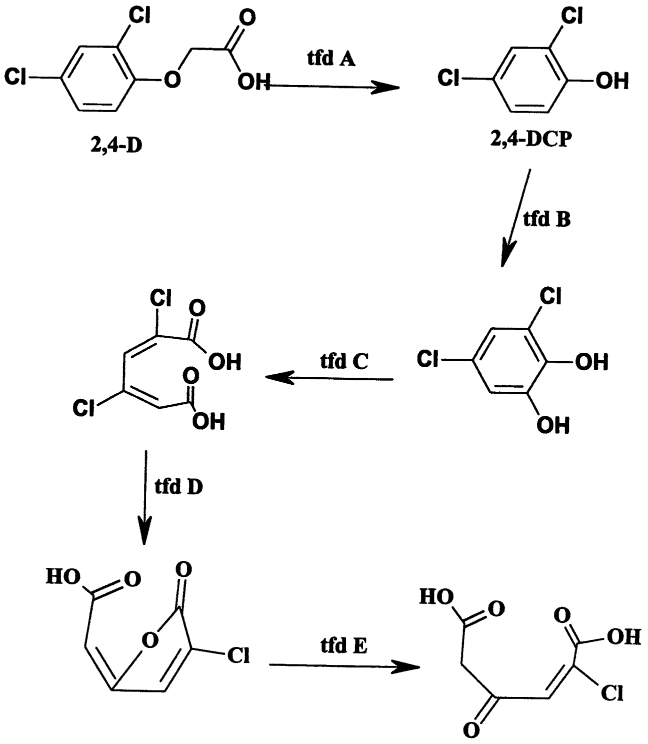

2,4-D
tfd A
2,4-DCP
tfd B
tfd C
tfd D
tfd E

Figure 10. Metabolism of 2,4-D by Alcaligenes eutrophus.

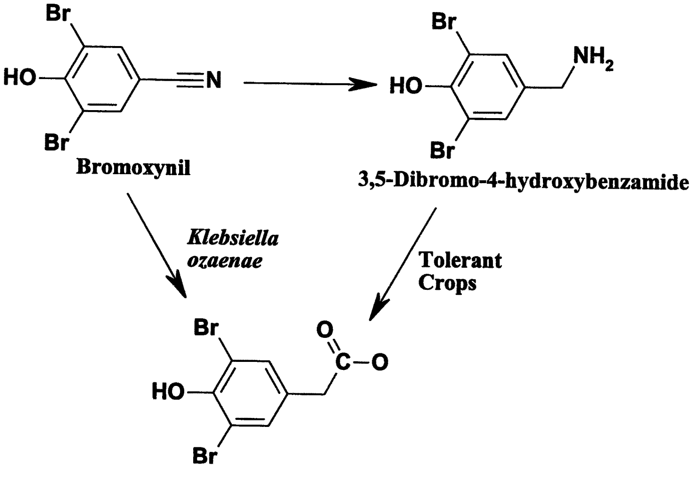

Bromoxynil
3,5-Dibromo-4-hydroxybenzamide
Klebsiella ozaenae
Tolerant Crops
3,5-Dibromo-4-hydroxybenzoic acid

Figure 11. Metabolism of bromoxynil.

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch019

The transformed cotton displayed high levels of tolerance to field application of bromoxynil and metabolized bromoxynil to 3,5-dibromo 4-hydroxybenzoic acid. Untransformed cotton did not metabolize bromoxynil (*33*). Transformed cotton plants containing this trait can tolerate up to ten times the use rate of bromoxynil under field conditions.

Elite cotton varieties were developed containing the bromoxynil tolerant trait and were introduced in 1997 by Stoneville Pedigreed Seed (Stoneville, MS.). Bromoxynil tolerant canola (*Brassica napus*), sugar beets (*Beta vulgaris*) and potatoes (*Solanum tuberosum*) have also been developed containing the same tolerance gene, and these new varieties will soon be commercially released.

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch019

Glufosinate

Glufosinate (2-amino-4-(hydroxymethylphosphinyl)butanoic acid) is a broad spectrum, postemergent herbicide with no crop selectivity. The successful strategy for producing glufosinate-tolerant crops was achieved by altering the plant's ability to detoxify the herbicide (*37*). Glufosinate is a potent, irreversible inhibitor of glutamine synthetase (GS) and is a truncated synthetic version of bialaphos; a natural product produced by *Streptomyces hygroscopicus.* Bialophos is a tripeptide (phosphinothricinyl-*L*-alanyl-*L*-alanine) that is metabolized to phosphinothricin within microorganisms and plants via cleavage of the two terminal alanine residues. Phosphinothricin is also a potent inhibitor of GS and has antibiotic activity (*37*).

The microorganisms that produce bialaphos protect themselves from the toxic effects of the subsequently produced phosphinothricin via metabolism. These microbes have a gene, which encodes for an acetyltransferase that can acetylate both phosphinothricin and glufosinate (Figure 12). Acetylated glufosinate is no longer a GS inhibitor. Two genes for this acetyltransferase have been isolated, the *pat* gene from *Streptomyces viridochromogenes* and the *bar* gene from *S. hygroscopicus* (*37*). Both of these genes have been used to transform various crops, and transformants expressing the acetyltransferase gene are highly tolerant to both glufosinate and phosphinothricin. The level of expression, which is needed for tolerance, can be very low. Tobacco transformed with the *bar* gene was completely tolerant to 4 to 10 times the use rate of glufosinate in the field (*38*). Transformants expressing the *bar* gene at levels as low as 0.001% of the total leaf protein were completely resistant. Field testing of crop varieties carrying either the *bar* or *pat* gene has shown that this trait is stable and there is no loss of agronomic characteristics. The trait is inherited as a single, dominant gene (*37*).

The *bar* gene has been successfully inserted into wheat, rice (*Oryza sativa*), maize, canola, sugar beets and sorghum (*Sorghum bicolor*). Glufosinate-tolerant canola was commercially introduced into Canada in 1996 and tolerant maize varieties were introduced in the U.S.A. in 1997. Glufosinate-tolerant soybeans and sugarbeet varieties will be introduced in the near future.

Glyphosate

Glyphosate (*N*-(phosphonomethyl)glycine) is a broad spectrum, postemergent herbicide that has no natural crop selectivity. Glyphosate-tolerant crops have been developed by transforming plants with a gene from an *Agrobacterium sp. CP4* encoding for a glyphosate-insensitive 5-Enolpyruvylshikimate-3-phosphate (EPSP) synthase (*39*). This approach has been highly successful in soybeans, maize and cotton, but the level of tolerance supplied by this gene is not sufficient in canola.

An alternative approach to make a crop tolerant to glyphosate is through altered metabolism. Most plants do not metabolize glyphosate to any great extent (*39*). Glyphosate can be metabolized by cleaving the C-P bond via a C-P lyase pathway. However, studies on this pathway in *Pseudomonas* spp. and *Eschericia coli* showed that this pathway was very complex and involved a number of different enzymes, so no further work was done (*39*). Glyphosate is also rapidly broken down by soil microorganisms to glyoxylate and aminomethylphosphonate (AMPA) (Figure 13), (*39*). Although several gram-negative and gram-positive bacteria have been identified which can metabolize glyphosate, Barry et al. (*40*) successfully cloned a metabolizing gene from *E. coli* strains that could utilize AMPA as a phosphorus source. They isolated a gene that encoded for glyphosate oxidase (*GOX*) that catalyzes the cleavage of the C-N bond in glyphosate, producing glyoxylate and AMPA (*39*).

This gene was put into a construct that is highly and constitutively expressed in plants. Since glyphosate rapidly translocates from treated leaves to meristematic points where the herbicide exerts it activity, rapid metabolism has to occur in treated leaves to prevent the herbicide from reaching the growing points. Initial attempts to transform tobacco with the *GOX* gene resulted in very low levels of expression. This problem was alleviated by fusing the GOX gene with the chloroplast transit peptide from the Rubisco small subunit, which resulted in a 10-fold increase in the steady state level of *GOX*. This version of *GOX* has been introduced into several different crop plants and the transformed plants show excellent tolerance to glyphosate under field conditions (*40*). Currently, glyphosate tolerant canola that is sold in Canada contains the *GOX* gene along with the *CP4* EPSP synthase gene.

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch019

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch019

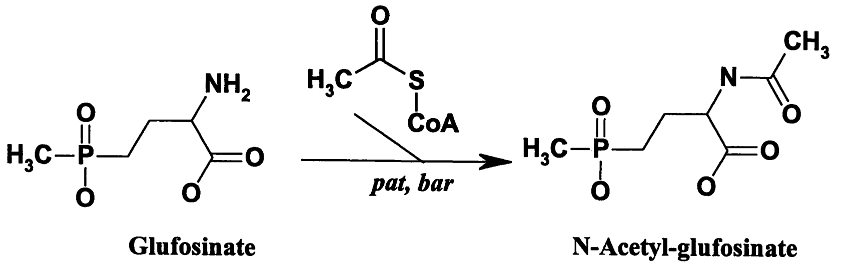

Glufosinate
pat, bar
N-Acetyl-glufosinate

Figure 12. Metabolism of glufosinate.

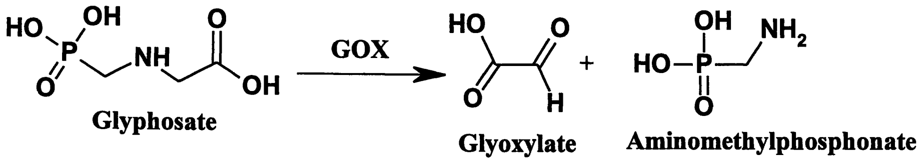

Glyphosate
GOX
Glyoxylate
Aminomethylphosphonate

Figure 13. Metabolism of glyphosate.

Summary

In summary, we have tried to illustrate how plants are able to metabolize herbicides by a variety of pathways with exceptional species diversity. It appears that the enzymes responsible for these differential routes of metabolism exist in multiple forms with little overlap in substrate utilization. Minor structural alterations of these compounds can result in dramatic and unpredictable changes in the rate and routes of metabolism. Thus, just introducing substituents (e.g. alkyl, halogens, etc.) into a compound that could be sites of metabolism may or may not result in crop selectivity because one cannot predict the whether or not these sites will be recognized by metabolizing enzymes. The major problem is lack of knowledge of the substrate affinities and specificity of individual plant enzymes, which makes it very difficult to predict metabolizable substrate structures with any certainty. Thus, one needs to make a large number of analogs with the different substituent patterns to determine if any will be detoxified. On the other hand, genetic engineering approaches to modifying the crops to tolerate the herbicide by a number of possible mechanisms have been successful. This alternative approach may be extensively exploited in the future, as the discovery of new crop selective, low use rate herbicides with novel modes of action becomes more difficult.

Literature Cited

1. Hatzios, K. K.; Penner, D. In *Metabolism of Herbicides in Higher Plants;* Burgess, MN, 1982, pp 15-92.
2. Beyer, E. M.; Duffy, M. J.; Hay, J. V.; Schlueter, D. D. In *Herbicides Chemistry, Degradation and Mode of Action*; Kearney, P. C.; Kaufman, D. D., Eds.; Dekker, New York, 1987, Vol. 3, pp 117-189.
3. Brown, H. M.; Fuesler, T. P.; Ray, T. B.; Strachan, S. D. In *Pesticide Chemistry. Advances in International Research, Development and Legislation. Proc. 7th Int. Congr. Pestic. Chem.*, Frehse H., Ed.; VCH, Weinheim, Germany, 1991, XIV, pp 258-266.
4. Lamoureux, L.; Shimabukuro, R. H.; Frear, D. S. In *Herbicide Resistance in Weeds and Crops*, Caseley, J. C.; Cussans, G. W.; Atkin, R. K., Eds.; Butterworth-Heinemann, Oxford, UK, 1991, pp 227-261.
5. Redina, A. R.; Felts, J. M. *Plant Physiol.* **1988**, *86*, 983-986.
6. Walker, K. A.; Ridley, S. M.; Lewis, T.; Harwood, J. L. *Biochem. J.* **1988**, *254*, 307-310.
7. Los, M. In *The Imidazolinone Herbicides*; Shaner D. L.; O'Connor S. L.,Eds.; CRC Press, Boca Raton, FL, USA., 1991, pp. 2-14.

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch019

8. Ladner, D. W. In *The Imidazolinone Herbicides;* Shaner D. L.; O'Connor S. L., Eds.; CRC Press, Boca Raton, FL, USA, 1991, pp 31-51.
9. Stidham,M. A.; Singh, B. K. In *The Imidazolinone Herbicides;* Shaner D. L.; O'Connor S. L., Eds.; CRC Press, Boca Raton, FL, USA.,1991, pp 71-90.
10. Tecle, B.; Da Cunha, A.; Shaner, D. L. *Pestic. Biochem. Physiol.* **1993**, *46*, 120-130.
11. Shaner, D. L.; Mallipudi, M. In *The Imidazolinone Herbicides;* Shaner D. L.; O'Connor S. L., Eds.; CRC Press, Boca Raton, FL, USA,1991, pp 91-102.
12. Brown, M. A.; Chiu, T. Y.; Miller, P. *Pestic. Biochem. Physiol.* **1987**, *27*, 24-29.
13. Brown, H. M.; Dietrich, R.F.; Kenyon, W.H.; Lichtner, F.T. *Proc. Brighton Crop Prot. Conf.-Weeds*, **1991**, *2*, 847-856.
14. Brown, H. M. *Pest. Sci.* **1990**, *29*, 263-281.
15. Brown, H. M.; Kearney, P. C. In *Synthesis and Chemistry of Agrochemicals II: Plant Biochemistry*; Baker, D. R.; Fenyes J. G.; Moberg, W. K., Eds.; ACS Symposium Series 443, Washington D.C., American Chemical Society, 1991, pp 32-49.
16. Diehl, K. E.; Stoller, E. W.; Barrett, M. *Pestic. Biochem. Physiol.* **1995**, *51*, 137-149.
17. Frear, D. S.; Swanson, H. R.; Mansager, E. R.; Wein R. G. *J. Agric. Food Chem.* **1978**, *26*, 1347-1351.
18. Frear, D. S.; Swanson, H. R.; Tanaka, F. S. *Pestic. Biochem. Physiol.* **1993**, *45*, 178-192.
19. Schuler, M. A. *Critical Reviews in Plant Science* **1996**, *15*, 235-284.
20. Hardcastle, W. S. *Weed Res.* **1974**, *14*, 181-184.
21. Hardcastle, W. S. *Weed Sci.* **1979**, *27*, 278-279.
22. Barrentine, W. L.; Edwards Jr. C. J.; Hartwig, E. E. *Agron. J.* **1976**, *68*, 351-353.
23. Barrentine, W. L.; Harwig, E. E.; Edwards, Jr. C. J.; Kilen, T. C. *Weed Sci.* **1982**, *30*, 344-348.
24. Mangeot, B. L.; Slife, F. E.; Rieck, C. E. *Weed Sci.* **1979**, *27*, 267-269.
25. Hartwig, E. E.; Barrentine, W. L.; Edwards, Jr., *J. Crop Sci.* **1980**, *20,* 825.
26. Lyon, B.; Cousins, Y. L.; Llewellyn, D. J.; Dennis, E. S. *Trans. Res.* **1993**, *2,* 162-169.
27. Wauchope, R. D.; Buttler, T. M.; Hornsby, A. G.; Augustijn-Beckers, P. W. M.; Burt, J. P. *Rev. Envrion. Contam. Toxicol.* **1992,** *123*, 1-155.
28. Llewellyn, D.; Last, D. In *Herbicide Resistant Crops;* Duke, S.O., Ed.; CRC Press: Boca Raton, FL, 1996, pp 159-174.
29. Don, R.; Pemberton, J. *J. Bacteriol.* **1981**, *145*, 681-686.
30. Streber, W.; Timmis, K.; Zenk, M. *J. Bacteriol.* **1987**, 169, 2950-2955.

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch019

31. Fukomori, F.; Hausinger, R. P. *J. Bacteriol.* **1993**, *175*, 2083-2086.
32. Bayley, C.; Trolinder, N.; Morgan, M.; Quisenberry, J. E.; Ow, D. W.; Ray, C. *Theor. Appl. Genet.* **1992**, *83*, 645-649.
33. Stalker, D. M.; Kiser, J. A.; Baldwin, G.; Coulombe, B.; Houck, C. M. In *Herbicide Resistant Crops;* Duke, S.O., Ed.; CRC Press: Boca Raton, FL, 1996, pp 93-105.
34. Buckland, J. L.; Collins, R. F.; Pullin, E. M. *Pestic. Sci.* **1973**, *4*, 149-162.
35. McBride, K. E.; Kenny, J. W.; Stalker, D. W. *Appl. Environ. Microbiol.* **1986**, *52*, 325-330.
36. Stalker, D. M.; McBride, K. E. *J. Bacteriol.* **1987**, *169*, 955-960.
37. Vasil, I. K. In *Herbicide Resistant Crops;* Duke, S.O., Ed.; CRC Press: Boca Raton, FL, 1996, pp 85-91.
38. DeBlock, M.; Botterman, J.; Vanderwiele, M.; Dockz, J.; Thoen, C.; Gossele, V.; Movva, N. R.; Thompson, C.; Van Montagu; M.; Leemans, J. *EMBO J.* **1987**, *6*, 2513-2518.
39. Padgette, S. R.; Re, D. B.; Barry, G. F.; Eichholtz, D. E.; Delannay, X.; Fuchs, R. L.; Kishore, G. M.; Fraley, R. T. In *Herbicide Resistant Crops;* Duke, S.O., Ed.; CRC Press: Boca Raton, FL, 1996, pp 53-84.
40. Barry, G.; Kishore, G.; Padgette, S.; Taylor, M.; Kolacz, K.; Weldon, M.; Re, D.; Eichholtz, D.; Fincher, K.; Hallas, L. In *Biosynthesis and Molecular Regulation of Amino Acids in Plants.* Singh, B. K., Flores, H. E.; Shannon, J. C., Eds.; ASPP, Madison, WI, 1992, pp 139-145.

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch019

Downloaded by UNIV MASSACHUSETTS AMHERST on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch020

Chapter 20

Non-Traditional Concepts of $ynergy for Evaluating Integrated Pest Management

Micheal Owen[1] and Jonathan Gressel[2]

[1]Agronomy Department, Iowa State University, Ames, IA 50011
[2]Plant Sciences, Weizmann Institute of Science, Rehovot, Israel

Synergy is measured by analyzing if the pest control efficacy of a mixture of chemicals applied together had a mathematically greater effect on a pest than the sum of the components applied separately. This methodology can also highlight synergies of little agricultural significance. We propose a new term "$ynergy" implying the importance of measuring the agronomic and economic benefits of chemical mixtures to the crop. $ynergy can be assessed by comparing chemical costs and other inputs for pest management separately, and in integrated packages, and by measuring the value of increasing the crop yield. $ynergy is used herein to measure the economic synergy obtained with various IPM practices compared with "stand alone" strategies. $ynergy allows the best estimation of the long-term value of integrated practices for pest management because it integrates the complexity of pest spectra, rotations, and field practices.

The importance of pesticide interactions in agriculture has been studied at length in numerous crops, pest species, and environments. These interactions have been described mechanistically, biologically, and biochemically. Generally, agriculture has become too dependent on chemically based, "stand alone" pest control methods, typically in monoculture systems. This has lead to enhanced pest problems and a reduced economy. Too often, the solution has been to adopt another stand-alone procedure when specific problems ensued.

The concept of using chemical mixtures was developed to control specific species. Various methodologies were developed to ascertain whether there was "just" an additive, or preferably a synergistic interaction. Chemical mixtures were reluctantly used when one component did not control a major pest in the spectrum of species. Many researchers have generated an incredible amount of data describing the interactions of agriculturally important chemicals, and these data, in turn, have been reviewed by others who have attempted to describe and predict the resultant interactions (*1, 2, 3, 4, 5, 6, 7*).

However, little effort was made to determine whether such mixtures were meaningfully synergistic, because the concept of synergy and the equations for measurement were predicated on single species effects. Depending on the specific interaction and pest/crop complex, these interactions may have significant agronomic importance. On occasion, the outcome has been useful discussions about maximizing efficiencies of herbicide mixtures using expert systems that deal with the total pest spectrum present (*8*).

These discussions are important and useful but do not go far enough. They do not integrate chemical control strategies separated in time that are used to control different species, other pest control measures, or consider the long-term effects of all pest management practices. For example, while continuous no-till production systems allow certain perennial weeds to proliferate while standard cultivation practices promote annual weeds, how do you compare using each alone or in rotation? We are beginning to relearn what was well known a few generations ago; that there is a distinct advantage to crop rotation (*9*). This advantage is especially apparent in our context when pesticides and types of cultivation are rotated with crops, particularly with crops from different families, varietal morphotypes and phenologies. This suppresses the whole pest spectrum and allows simpler pest control in each crop.

The possibility of synergistic interactions between chemicals and other agronomic practices was never considered, nor were the long-term implications of synergies considered. A synergistic interaction has great potential to improve crop production efficiency by lessening environmental and economic impact (*10, 11*). However, traditional definitions and expressions of synergy may not be appropriate in the changing world of agriculture. The mathematics of measuring synergy (*1, 6, 7, 12, 13*), which demonstrates minuscule synergistic interactions, can be agriculturally irrelevant, even when fully endorsed by statisticians.

Such mathematics were inadequate for measuring anything but the effects of simultaneous use of one vs. more than one pesticide on a single pest species. Limiting discussions of synergy to a single pest, biochemical reaction, or physiological measurement is likely to lessen the utility of the phenomenon to agriculture. Synergy from sequential or multiple pest management strategies and resultant effects on the whole pest system cannot be calculated. Given the concerns expressed by the public sector about agriculture and the significant changes in agriculture, it is appropriate to redefine synergy and demonstrate how synergistic interactions can serve to improve agriculture.

The ultimate goal of the farmer is cost-effective crop production resulting in the highest obtainable economic yield. Despite this, the crop is typically ignored

Downloaded by UNIV MASSACHUSETTS AMHERST on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch020

in traditional synergy measurements, other than assuming no direct phytotoxicity results from chemical treatments. A mixture of compounds could synergistically control one pest, but open a niche to other pests resulting in crop damage (e.g., by removing a beneficial insect that a single insecticide would have not controlled). While being a synergy by conventional concepts, this is actually an antagonism. In this example, the potential crop response would be an indirect yield reduction by the mixture due to a new pest outbreak.

With so many variables, how can we calculate synergies in a manner useful for agriculture? How do we put this in an equation dealing with pests? Non-monoculture agronomic systems are indeed more complex, but the measurement of synergy for pest control can actually be simplified if all effects are integrated economically, and termed "$ynergy" (*14*). There is "$ynergy" when the cost of using multiple pest control strategies, in concert, is less (per unit crop yield) than the cost of pest control using the individual strategies. More simply stated, when a combination of treatments gives more cost-effective, profitable pest control and thus enhances crop yield when compared with separate stand-alone treatments, there is $ynergy. This paper reviews the current understanding of synergy, compares it to $ynergy, and describes how the interaction of pest control strategies might be considered in future agricultural systems.

Downloaded by UNIV MASSACHUSETTS AMHERST on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch020

Pest-based, Classical Definitions of Synergy

Understanding the classical definitions for synergy allows a better understanding of the implications of our crop-based definition: A synergistic mixture is one that has a greater pesticide effect than expected from the sum of each component used separately. Researchers usually define the "sum of the components" as the rates of the components used. However, the farmer defines synergism based on the net return compared to the costs.

Types of Synergy

Pesticide synergy can be of three types: a) where one component of the mixture is a non-toxic adjuvant (assuming a literal definition of adjuvant) allowing a lower rate of the second component to be used; b) where both components are toxic, but the combination allows lower levels of both to be used; and c) where neither component is toxic, but the combination is toxic (a coalitive synergy). The boundaries differentiating the three types are not always as clear as the definitions may imply. An adjuvant may be toxic at a high concentration and a pesticide nontoxic at the low concentration used in a mixture. Furthermore, a pesticide may act as an adjuvant.

As simple as the definition may appear, the literature is replete with various semantic and mathematical definitions of synergy (*1, 3, 4, 5, 6, 7, 15, 16*). We will not try to differentiate between the terms often used: "synergism", "more than additive", "potentiation", and "enhancement effect" since they all meet the requirements defined for synergists.

Synergy and the Single Pest

There are many reports of synergistic pesticide interactions increasing the effect on single pest species. Typically, these descriptions of synergy focus on the uptake, translocation, or metabolism of a particular pesticide in response to another pesticide or putative synergist. Alternatively, simple kill rate, weed biomass measurement, or visual estimation of "control" may be used to mathematically calculate if a chemical mixture behaves synergistically. The response of the crop is not a factor considered important with the description of synergy. This section will provide a brief overview of synergy reports in the literature.

Unfortunately, there is considerable variability in weed response to herbicide combinations. A herbicide combination that is determined to be synergistic for one weed species may be antagonistic for another weed species. Importantly, those mixtures that are synergistic for specific weeds may also demonstrate increased activity on the crop species. On rare occasions, research has addressed other factors such as the impact of environmental conditions, plant size, or age on synergy.

While many herbicide combinations are potentially synergistic, other agricultural chemical interactions such as antagonisms should not be ignored. For example, Byrd and York (*17*) studied interactions between sethoxydim and several insecticides and found that combinations with carbaryl inhibited large crabgrass (*Digitaria sanguinalis*) control. However, there are examples of synergy with other agricultural chemicals. Synergistic responses of sicklepod (*Cassia obtusifolia*) to the insecticide toxaphene in combination with acifluorfen or 2,4-DB were observed (*18*).

Environment can influence the type of response, and field responses can differ from those in the laboratory or greenhouse. The interaction of toxaphene and acifluorfen was synergistic when measured under field conditions but was additive when the experiment was conducted in the greenhouse (*18*). Conversely, tridiphane acted synergistically in the greenhouse increasing wild oat (*Avena fatua*) control with atrazine or cyanazine (*19*). The response of redroot pigweed (*Amaranthus retroflexus*) to acifluorfen and bentazon was synergistic in the field but antagonistic in the greenhouse (*20*). A synergism was reported between the fungicide benoxazole and both chlorbromuron or simetryne herbicides for the control of the weed barnyardgrass (*Echinochloa crus-gali*) in paddy rice, but the efficacy of the photosynthesis inhibitors was reduced as the grass weed aged (*21*).

Synergies in weed control can also affect the crop by increasing phytotoxicity to the crop or by protecting the crop from the herbicide

Downloaded by UNIV MASSACHUSETTS AMHERST on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch020

combination. There was a synergistic interaction between atrazine and 2,4-D when applied with various crop oil adjuvants in controlling weeds (*22*). However, phytotoxicity to sweet corn (*Zea mays*) was also increased. Velvetleaf (*Abutilon theophrasti*) control with acifluorfen was improved when a 10-34-0 fluid fertilizer was added to acifluorfen, and phytotoxicity to soybean was reduced by the addition of the fluid fertilizer (*23*). However, different growing conditions caused different levels of synergism. The synergism on velvetleaf was also a function of weed size; greater synergism was found on larger plants (*24*).

Nicosulfuron and rimsulfuron in a 1:1 mixture were synergistic for the control of smooth crabgrass (*Digitaria ischaemum*), but not common ragweed (*Ambrosia artemisiifolia*) (*25*). The effect of these herbicides on corn (*Zea mays*) was additive, yet antagonistic on soybean. Blackshaw (*26*) reported a synergistic interaction with ethametsulfuron-methyl and clopyralid for the control of common lambsquarters (*Chenopodium album*) and redroot pigweed. Other weeds did not respond to the herbicide interaction, yet oilseed rape was tolerant to both herbicides.

Synergy can be due to the inhibition of herbicide metabolism and conjugation (*27*), although the response can vary depending on weed species (*28*). Synergy may occur due to differential protein synthesis (*2*), herbicide uptake (*29, 30, 31, 32*), translocation (*32, 33)* or reduced singlet oxygen scavenging (*34*). In some instances, no explanation for a synergistic interaction was provided (*35*, 36).

Legal Synergy

Patent offices have accepted three types of data as synergism (*37*): (a) The more than additive response, when two compounds give partial control separately, and when they give more control together than with the sum of each separately. This is easiest to prove when neither, or only one component, has no effect (i.e., as with coalitive synergism or with an adjuvant). Synergism is problematic to demonstrate when the sum would have to exceed 100% control. (b) The control provided by the second component of a mixture exceeds the fractional control it would be expected to give (i.e., if 0.5 kg ha^{-1} of pesticide A gives 70% control and 0.5 kg ha^{-1} B gives 50% control, additivity suggests 120% control). Instead, according to Limpel's formula used by many patent offices (*37*), component B should give more than a 50% control of the 30% of pests uncontrolled by A; (i.e., control must exceed 0.7 + (0.5 X 0.3) = >85% control).

Limpel's formula seems to lack both common sense as well as mathematical logic, because of the lack of linear additivity. Still there may be cases were a modification of Limpel's formula is relevant. Some biocontrol agents individually do not provide adequate control regardless of amount used (Figure

Downloaded by UNIV MASSACHUSETTS AMHERST on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch020

1). However, when two biocontrol agents are mixed and full control is achieved, this seems linguistically or intuitively to be synergistic, even if the effects are only additive mathematically. Thus, the combination of biocontrol agents meets Limpel's requirement for synergy as well as agricultural need.

This lack of complete control irrespective of rate is more likely to be seen in the field than in the laboratory. Pest populations in the laboratory are typically synchronous. Pest populations in the field are typically asynchronous and the field may present other problems. For example, two insecticides might control different and mutually exclusive instars, two herbicides may control weeds at different stages of growth, or there could be synergistic interactions between a surface active and a systemic fungicide or insecticide. (c) A test that overcomes the non-linearity of dose response curves simply states that a given rate of mixture is better than the same rate of either component. Agronomically, this makes the most sense and is probably of most value. Thus, if a mixture of 250 g ha^{-1} pesticide A with 750 g ha^{-1} of pesticide B provides "adequate" pest control, the interaction is defined as synergistic when 1 kg ha^{-1} of either component separately does not provide this level of control.

Downloaded by UNIV MASSACHUSETTS AMHERST on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch020

Mathematical Synergy

The simplest mathematical determination of synergy is to plot the isobole, which is the concentration of each component in the presence and absence of the other resulting in an equal (fixed) response (*7, 12, 13, 15, 38, 39, 40, 41, 42*). The isobole will be a straight horizontal line at the effective concentration of the pesticide in the mixture with an adjuvant that by itself has no synergistic effect.

If two pesticides are mixed, isobole slopes showing antagonism, additivity, or synergism can be achieved. The theoretical additive line is used as the null hypothesis for an "additive dose model". Any curve beneath this line denotes synergy and above this line denotes antagonism (*12, 15, 38, 43, 44*). It is obviously impossible to plot the slope for a coalitive synergy because separately there is no effect.

In contrast, "multiplicative survival models" are considered superior (though more cumbersome) by many statisticians (*1, 6, 7, 12, 13, 43*). Survival of organisms in response to chemicals is expressed as a proportion. The proportions of survival from each chemical used separately are compared with the proportional survival in response to chemical mixtures. The actual models used and the statistical techniques necessary to show synergism can provide statisticians with much grist even when the actual synergism is not great. Morse (*6*) used probit plots for both additive and multiplicative models. She found it harder to determine a synergy than had the experimentalists and points to the problems of discerning weak synergies and the need for a considerable amount of data to do so.

The major practical problem using isoboles to describe synergy is that they are plotted using data of little agronomic significance. Statistically, it is most

Downloaded by UNIV MASSACHUSETTS AMHERST on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch020

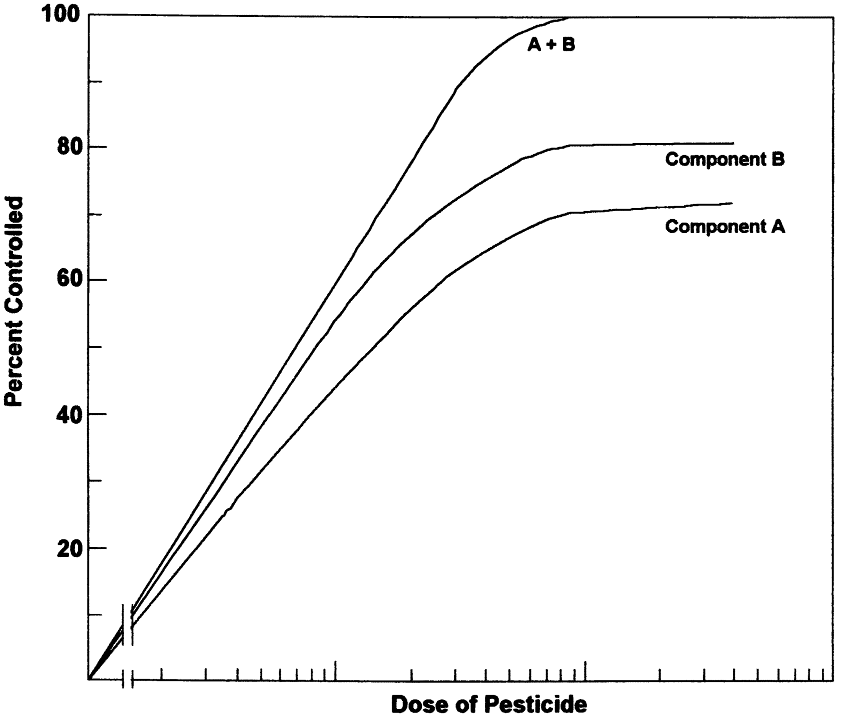


Figure 1. When neither pesticide can control the pests, irrespective of rate, is it synergy when together they can?

accurate to plot isoboles from some point on the slope of a dose response curve; i.e., some point between the LD_{20} and the LD_{80}. Conversely, the farmer is rightfully interested in some point between the LD_{95} and LD_{99}, which can only be shown by extrapolation. From the farmer's point of view, the combination of two sublethal pesticide rates must provide "adequate" pest control, or it is of little value.

Proposed New Definition of $ynergy

Researchers have stressed the need for applicability of synergistic interactions toward real-world agricultural problems and implied that through an understanding of synergy, economic and environmental benefits could be achieved (*4, 43*). However, there was little to demonstrate that the synergy was actually a useful consideration for growers because current concepts reflect only changes in the pest and not the crop. In a review of models developed to evaluate and present the interactions of pesticides, it was concluded that all methods had difficulty in expressing response to pesticides (*45*). Importantly, the data upon which these models were based was the growth response of the pest and the models did not describe the overall impact of the interaction on the agricultural system. The lack of consideration of crop response makes these models of little agronomic use.

Given the current economic and environmental concerns in agriculture, there is a need to improve pesticide utilization. Ideally, a crop-based concept of $ynergy should consider other factors to be useful, including concerns about the development of pesticide resistant populations, pest thresholds, crop phytotoxicity, and economic risk management (*46*).

$ynergy and Environmental Considerations

Risk indices that compared the environmental concerns due to pesticide use with the profitability for several crop production systems, showed that environmental risk was manageable without significant economic loss (*47*). We suggest that by evaluating $ynergy in a different, more field-applicable manner, growers will realize greater pest management benefits while accommodating concerns for the environment.

$ynergy and Biological Considerations

Clearly any use of $ynergy will have to be field-based, without over-generalization since $ynergies vary depending on pest and climatic conditions. For example, weed thresholds based on crop yield losses and weed seed

Downloaded by UNIV MASSACHUSETTS AMHERST on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch020

production are considerably lower if weed seed production is considered (*48*). There was considerable variation in the effect johnsongrass (*Sorghum halepense*) control had on soybean (*Glycine max*) production economics attributable to climatic conditions as well as production system (*49*). Many aspects of soybean grain quality and yield were reported, and a general assessment of weed control correlated linearly with the net return on investment. Thus, the prediction of herbicide activity might be sufficient to accommodate an understanding of risk. However, herbicide activity is strongly influenced by climatic conditions, and thus the predictability of weed response to herbicides is not consistent (*50*). Methods to determine the synergism of additives on herbicides applied postemergence had the same shortcomings (*5*). Crop injury and weed control have been used together to describe herbicide interactions (*51*) and biochemical descriptions of synergism have also been used (*52*). In each case, however, there is a need for more information and applicability of the interaction on agriculture.

Clearly, descriptions of synergy should be crop oriented, include tangible measurements of economics and risks, and provide the grower with a strong tool with which to make pest management decisions. The new concept of $ynergy is based on the yield benefit from using various pest management strategies. Yield assessment could include yield quality and quantity which is reflected in market price. It would be good if environmental costs could be included in the new concept, but to date, this does not appear feasible. Furthermore, any new concept should account for the response of pests and pesticides to changing weather conditions. Unless this is possible, the utility of the new measurement/assessment of $ynergy is compromised.

Synergy Versus $ynergy

The various algebraic descriptions used to determine synergy, while scientifically useful, have not been utilized effectively in agriculture. Greater utility could be achieved by measuring a chemical interaction with due consideration to the profitability of crop production. Assessing $ynergy by examining crop yield or economic gain would provide agriculture with a better means to utilize synergistic interactions of pesticides involved with pest management.

Advantages of $ynergy

The major advantage of $ynergy is that measurements are based on crop profitability and not on pest control *per se*. The simple profit measurement of $ynergy does not require that the components be just chemicals; $ynergy between chemicals and cultivation (e.g., banded herbicide application with inter-row

Downloaded by UNIV MASSACHUSETTS AMHERST on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch020

cultivation) could be measured. A more modern $ynergy could be between scouting or remote sensing of pest patches, followed by post-emergence pesticide application to the pest patch (*53, 54*). More importantly, $ynergy could be assessed over a multi-year average of economic returns, just as farmers are learning to do with other aspects of crop production.

$ynergy and Risk Management

One essential consideration for the application of $ynergy is the relative risk that producers must accept when they use this concept in pest management decisions. Risk management by growers is of increasing importance as agriculture changes. However, often a grower manages risk in a manner that does not follow scientific or economic rationale. For example, weeds are controlled at levels beyond those that are economically important with regard to potential yield reduction due to competition. Growers justify these management decisions by suggesting the need to eliminate weed seed production, however often the amount of weed seed returned to the seedbank was minimal and not economically warranted.

Farmers typically avoid or attempt to minimize risk when making pest management decisions (*55*). Farmers expect some acceptable level of return if they have to make decisions that include some acceptance of risk (*56*). The lack of ability to estimate risk complicates decision making by growers. The risk associated with pesticide treatment failure is a major consideration for farmers and may or may not be associated with any biologically-based data. Furthermore, the presence of pests in a field may represent a significant risk for growers, whether or not the population density is sufficient to cause any significant economic loss of yield potential.

Importantly, while growers may make decisions based on the economics of $ynergy, they also make risk decisions based on time availability and labor constraints (*57*). For pest management decisions to be based on $ynergy, growers must consider the risk of timeliness of the practice and whether or not they have the time available to conduct the strategy. The latter reflects a number of other management considerations and environmental factors, notably soil moisture. Better management decisions and decreased risk for the management strategies could be obtained if farmers had precise historical records of days available for fieldwork (*57*). Accounting for environmental factors would greatly enhance the utility of $ynergy as a basis upon which pest management decisions could be made.

$ynergy could be assessed by considering final pest population densities, weed seed production, risk management, and esthetics. However, like synergy, $ynergy has a limitation reflecting the potential lack of consistency of measured pest responses, whether biochemical or economic. The predictability of synergy and $ynergy, and thus their utility, are highly dependent upon the environment. As pest/crop interactions change in response to the environment, the importance

Downloaded by UNIV MASSACHUSETTS AMHERST on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch020

of chemicals or management strategy interactions, and the subsequent pest or crop responses, will also reflect the environmental conditions. The variability of $ynergy may be lessened by reviewing multi-year information. Thus, we believe that $ynergy better represents a useful tool for agriculture and profitability than does synergy. However the application of $ynergy may have some of the same shortcomings as synergy. Examples of $ynergy and the various forms growers might consider for incorporating this concept in their risk management planning for pest control are discussed below.

Downloaded by UNIV MASSACHUSETTS AMHERST on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch020

Case Histories of $ynergy

There are a number of possible interactions that could be considered $ynergistic. These include the interaction between pesticides, various application strategies and crop rotations. $ynergism could have specific applications such as for the management of pest resistance, the control of a particularly pernicious pest, or the management of some environmental risk such as soil erosion. However, the examples we provide are more general and reflect the management of weeds and resultant economics.

Effect of $ynergy on Resistance Management

The mixture of more than one chemical or procedure can affect pesticide resistance management, and if a compound that might be lost due to resistance has value, there can be a long-term $ynergy in keeping it available. If one compound in a mixture only weakens, but does not control a particular pest, it can help preserve the other compound in the mixture from extinction due to resistance. Whenever a rare resistant individual evolves, it is so unfit and non-competitive due to the second compound, the resistant individuals quickly disappear from the population. A large-scale epidemiological example of this can be seen with the mixtures of chloroacetamide herbicides (used primarily for grass control) and triazines (used mainly for control of broadleaf weed species). Triazine-resistant broadleaf weeds evolved in many locations and on millions of hectares where triazines were used alone. However the authors are not aware of resistant populations developing where triazines were used in mixtures with chloroacetamides, which only slightly debilitate most broadleaf weeds.

Mechanical and manual control can have the same $ynergistic effect. Models predicted that five witchweed (*Striga*) plants would appear per hectare in the first year of using imidazolinone herbicides on resistant corn varieties. If these were removed by hand before setting seed, there would be no problem from resistant populations (*58*). However, this likely represents an impossible goal.

$ynergy from Split Applications of Herbicides

$ynergy arising from split applications of herbicides is well-established and, in fact, the split application of herbicides represents a common weed management strategy. However, typically growers will include full rates of the particular herbicide used in each application and thus do not take advantage of possible $ynergisms that may result from split applications of the same compounds.

Influence of Climate on Split Applications

Climatic conditions influence the effectiveness of reduced herbicide rates and thus decrease the predictability of weed response to this strategy. Split applications of herbicides at reduced rates minimize this risk since weeds that are previously injured by a herbicide are more susceptible to a subsequent application. This likely improves the $ynergy attributable to split applications of herbicides. Sequential applications of quarter rates of herbicides provided control equivalent to a single application of the full herbicide rate (*59*).

Unfortunately, the use of split application strategies is inconsistent with the actual preferences of growers to use a single herbicide application for weed management. However, weed control from a single herbicide application is often variable, and changing weed populations, differential germination habit, input costs and environmental concerns have resulted in general acceptance of the split application approach (*60*).

Mechanisms for Split Applications

As herbicide resistant crops are becoming more widely used, split herbicide applications have become widely used in corn and soybeans and will probably increase in other crops. Crop competitiveness over weeds may be enhanced in response to split herbicide applications (*61*). Reduced herbicide rates in split applications also reduced weed seed production, albeit, inconsistently (*59*).

There are a number of ways that split applications of herbicides might impart $ynergy on a weed management system. Pretreatment with mefluidide enhanced the uptake, translocation and metabolism of acifluorfen applied sequentially in several weed species (*62*). Several thiocarbamate herbicides reduced the deposition of epicuticular waxes on cabbage leaves and enhanced the penetration and absorption of DNBP (*63*). Epicuticular waxes impede herbicide efficacy by reducing penetration into the leaf tissue; this action could be used to $ynergize herbicides applied sequentially to the thiocarbamates.

Several thiocarbamate herbicides stimulated the germination of velvetleaf and common lambsquarters seeds and could enhance control of these weeds by postemergence herbicides (*64*). Allelochemicals and herbicides could interact to increase the activity of the herbicides when used at reduced rates (*65*). Given the increase of conservation tillage systems in crop production and subsequent

Downloaded by UNIV MASSACHUSETTS AMHERST on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch020

higher residue levels left on the soil surface, this interaction could provide $ynergistically enhanced weed control.

Tridiphane was a metabolic synergist applied sequentially to triazine herbicides (*28, 66*). A likely $ynergistic interaction exists between residual herbicides and sequential applications of glyphosate at reduced rates for several weeds in soybeans (*67*). Control, relative to the glyphosate rate, was dependent on the environmental conditions, weed species and weed size. Sequential applications of herbicides at lower rates were equivalent to full rates for weed control and soybean yields (*68*). However, the use of lower herbicide rates can lead to "creeping resistance" due to multi-factor, polygenic type factors (*69, 70*).

There are examples of herbicides applied sequentially that interact antagonistically (*35, 71, 72*). Furthermore, field research data suggests the lack of consistent $ynergistic responses (*73, 74, 75*). Late glyphosate applications did not control velvetleaf or Pennsylvania smartweed (*Polygonum pensylvanicum*) while earlier applications did not control common waterhemp (*Amaranthus rudis*) or common cocklebur (*Xanthium strumarium*) (*76*). Sequential treatments generally controlled all weeds and thus demonstrated $ynergy. Clearly, some evaluation of risk is needed to take advantage of potential $ynergy that exists from split herbicide applications used for weed management, particularly when herbicide rates are reduced.

Economics of $ynergy from Split Applications

Possible $ynergy has been measured when sequential herbicide applications are compared to single strategies. Four sequential applications of glyphosate applied throughout the season yielded 150% more soybeans than metolachlor applied preemergence (*77*). Typical sequential applications of residual herbicide followed by postemergence herbicides had 54 and 95% yield increases. All of these sequential applications demonstrated $ynergy, which might not have been measured as synergy. Using an estimated herbicide application cost of \$12 ha^{-1} and a glyphosate cost of \$8 L^{-1}, the cost of the sequential treatment with the highest yield was \$64 ha^{-1}. The cost of the single application with the worst yield was approximately \$44 ha^{-1}. Assuming a price for soybeans of \$0.15 kg^{-1}, the net return for the worst treatment was \$193 ha $^{-1}$. The $ynergy for the best treatment, the application of four glyphosate treatments, was \$292 ha^{-1}.

While crop yield is a major factor when determining $ynergy, other factors (e.g., weed seed production) should also be considered. A 12% yield increase above a single residual herbicide treatment applied before planting for a glufosinate-tolerant soybean variety was demonstrated when the residual herbicide treatment was followed by a post-emergence glufosinate application (*78*). This represents an estimated increase in gross return of \$60 ha^{-1} for the sequential treatment. The $ynergy observed was \$7 ha^{-1} and included increased soybean yield and lower weed populations. The resulting lower weed seed production would likely increase the value of the treatment. Given grower concerns for future weed populations and the subsequent economic risk

Downloaded by UNIV MASSACHUSETTS AMHERST on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch020

associated with weed seed production, it is desirable to establish a manner by which the effect of weed management strategies on weed seed production could be included in a calculation of $ynergy.

Pest populations will affect the need for sequential applications. When weed populations are low or environmental conditions enhance the weed control for the initial herbicide treatment, sequential applications may be unnecessary and thus $ynergy does not occur. Furthermore, when yields or the crop price are low, better weed control from sequential applications may not $ynergistically improve yield. However, $ynergy does occur in many instances where yield increases were relatively low. Corn yields increased 10% above a residual herbicide application to the soil when a split application of glyphosate was used on resistant corn (*79*). At a corn price of \$0.08 kg^{-1}, the sequential application increased the gross return by \$72 ha^{-1} and demonstrated $ynergy. There was an 8% yield increase when split preemergence plus postemergence herbicide applications were compared to a single postemergence application of glyphosate in glyphosate-resistant corn (*80*). This represented a gross return difference of \$64 in favor of the split application and a net increase of approximately \$8 ha^{-1} of $ynergy. If the value of the crop were higher, these relatively low increases in yield would prove to be considerably more $ynergistic.

When weed populations are high, control from initial herbicide applications is poor, or when the environment favors multiple weed germination events, sequential herbicide applications will markedly improve crop yields and thus demonstrate $ynergy. There was a 34% increase in corn yield when a split treatment of a preemergence herbicide followed by an early postemergence herbicide was compared with single, early post-emergence treatments (*76*). The yield difference averaged >3.5 T ha^{-1} or a gross return of approximately \$262 ha^{-1} which represents a $ynergy of approximately \$208 ha^{-1} for the split application. An average yield increase of 96% due to a split herbicide application was measured in grain sorghum (*Sorghum bicolor*) (*81*). This represents considerable $ynergy over the application of one soil-applied residual herbicide.

$ynergy From Herbicide/Cultivation Interactions

Historically, mechanical weed control had been the backbone of weed management. However, from the grower's perspective, herbicide use and changing tillage practices have lessened the importance of mechanical weed management. Functionally, there should be few differences between a selective postemergence herbicide application and a selective mechanical strategy. Both strategies are subject to weed and crop size constraints and a requirement of timeliness for optimum impact on weed management (*60, 82*). Possible differences between mechanical and herbicidal weed management focus upon the potential for crop phytotoxicity attributable to a postemergence herbicide and the lack of in-row weed control from row cultivation. Furthermore, mechanical weed management may be subject to greater constraints imposed by the weather.

Downloaded by UNIV MASSACHUSETTS AMHERST on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch020

Risk and Mechanical Strategies

Mechanical weed control practices are typically more time, labor, and fuel intensive. Farmers may need to increase the size of equipment and expend more time to manage weeds if mechanical strategies are used to replace herbicides (*55*). Farmers may want to limit the crop area planted per day to allow for timely mechanical strategies (*57*). Also, field days available for mechanical weed management practices may be more limiting than for herbicide applications, given the importance of soil moisture conditions on mechanical practices (*55*). This risk should be accommodated in a calculation of $ynergy attributable to mechanical weed management practices.

Row width also has a significant impact on mechanical weed control. Weed control improved as row width narrowed for corn, soybean, and sunflower (*Helianthus annuus*) while requiring less mechanical and herbicide inputs (*83*). While it was assumed that this represented a long-term environmental benefit for agriculture, growers must also accept greater risk of variable results, given the importance of favorable weather conditions on the effectiveness of the system. The additional seed costs must be integrated into the calculation of $ynergy. Furthermore, narrow rows increase the critical nature of timely mechanical strategies and also increase the potential for crop stand loss attributable to poor technique both which may influence the level of $ynergy.

Consistency of response to mechanical strategies was a consideration with regard to crop yield and for final weed populations and that will affect the calculation of $ynergy. Weed populations in banded-herbicide applications were similar or less than broadcast applications in 92% of a five-year, multi-location study (*84*). Corn yields for the band applications were the same or greater than broadcast applications in 99% of the locations. Importantly, only 8% of the banded-herbicide treatments had higher weed populations than the broadcast applications 60 days after planting

$ynergy from mechanical weed control responds more to crop yield than other factors. Rotary hoeing, plus in-row cultivation controlled weeds similarly to control by herbicide treatments (*85*). Integrating reduced herbicide rates and mechanical tactics yielded greater economic returns for the integrated systems when compared with mechanical weed control as a single strategy (*86*). Importantly, grower risk was reduced by the integrated systems while environmental benefits still included a 50 to 75% reduction in herbicide use.

Economics of $ynergy from Mechanical Strategies

Mechanical weed control treatments had variable yields, resulting in better net returns for herbicide treatments (*87*). Weed cover and corn yields after banded-herbicide application plus cultivation were the same as broadcast herbicide treatments regardless of mechanical tactics, demonstrating $ynergy at standard costs (*88*).

Downloaded by UNIV MASSACHUSETTS AMHERST on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch020

Unfortunately, there has been little research published that allows the calculation of $ynergy attributable to the interaction of herbicide and mechanical weed management strategies, but some results are described below. Cultivation reduced late-season weed populations and improved yields when reduced herbicide rates were applied to soybean (*68*). Rotary hoeing did not reduce the weed density or improve soybean yield for band and broadcast herbicide applications (*89*). However, cultivation following preemergence or postemergence herbicide applications increased soybean yields and gross profit margins 80% of the time. The best gross margin, compared with the cost of weed control was for a broadcast postemergence herbicide followed by cultivation (*89*).

While $ynergy can be assumed to occur in the aforementioned research, the actual economic return is a critically important component of any management decision that a grower makes. Reduced herbicide rates were also included as a factor in a study comparing broadcast and band herbicide applications with and without cultivation (*90*). Generally, the inclusion of mechanical weed control tactics provided a $ynergistic increase in gross economic margin. Furthermore, total broadleaf weed biomass was reduced when cultivation was included with the herbicide treatment. If harvest efficiency, weed seed production, and "esthetic" considerations could be factored into the calculation of $ynergy, it is anticipated that the economic return would increase dramatically in favor of the mechanical management strategies.

Greater $ynergism was observed when herbicides were banded and cultivation included compared with broadcast applications. For example, a banded application of metolachlor plus atrazine at two rates, with and without cultivation, demonstrated a yield increase of 124% for the low rate and 101% for the high rate (*90*). However the calculated $ynergism was \$276 ha^{-1} for the higher rate compared with \$241 ha^{-1} for the lower rate, due to a higher corn yield attributable to better weed control. The yields for the same herbicide rates applied broadcast with cultivation, compared to no cultivation, were 4 and -3%, for high and low rates, respectively. This represented a $ynergism of only \$12 ha^{-1} and -\$24 ha^{-1}. A year later, a negative $ynergism was measured for the broadcast treatments while there was lower $ynergism for the banded-herbicide treatments, with cultivation at the same location. However, the banded treatments still allowed a 60% reduction in herbicide load to the environment and this savings should be factored in to the $ynergy. Unfortunately, there is no current way to calculate the economic returns for improvements to the environment.

Soybean yield increases were observed when cultivation followed banded applications of bentazon applied postemergence to control common cocklebur (*91*). While not consistent, $ynergy was generally greater for the reduced rates of bentazon. However, $ynergy was minimal or negative when bentazon rates and application strategies were compared without cultivation. Weed biomass was also reduced with the addition of cultivation to the banded-herbicide treatment when weed emergence was uniform. When climatic conditions did not

Downloaded by UNIV MASSACHUSETTS AMHERST on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch020

favor uniform emergence of common cocklebur, sequential applications of bentazon provided better weed control, yields, and enhanced $ynergy above the addition of cultivation. Thus, climatic conditions are important considerations when evaluating the $ynergy of cultivation with herbicides, regardless of the application strategy.

$ynergy From Crop Rotations

$ynergy attributable to crop rotation is difficult to measure and little research has been conducted that includes the treatments appropriate to allow the comparisons. However, the benefits of crop rotation on pest management, soil quality and potential yield are widely recognized. Improved yield when grain crops follow legume crops is a widely accepted response to this management strategy. Rotating crops with different life cycles is recommended as an important cultural pest management strategy. Importantly, $ynergy attributable to crop rotation may require several years to fully assess.

Different crop cultivars have different competitive abilities, potentially improving pest management when included in a rotation scheme whether dealing with weeds (*46*) or pathogens (*92, 93, 94*). Furthermore, different planting patterns for crops in rotation may improve competitiveness and provide $ynergy for weed control. Weed management could be improved with delayed planting and narrow row spacing appropriate for soybean production (*57*). Thus, including soybeans in a crop rotation system could provide some $ynergy to a weed management program. $ynergy may occur when the herbicides available for specific crops provide better control and thus lessen the weed populations for rotation crops.

The weed seed bank profile was changed in response to crop rotation strategy, which affected weed control and enhanced $ynergy (*95*). The $ynergy from the crop rotation would result from greater depletion of the active seed bank by high germination rates and the subsequent control of weed seedlings. The importance of $ynergy gained from a rotation of soybean with rice (*Oryza sativa*) was the improved control of red rice (*Oryza sativa*) (*35*). Herbicides available for soybeans had greater activity on this important weed, and thus red rice populations were reduced when soybeans were grown, making subsequent rice production on the same fields more profitable.

$ynergy from crop rotation can also occur when the rotated crops have a different growth habit that can negatively affect the weed population. When a perennial forage crop is included in a crop rotation, summer annual weed populations are reduced. Similarly, when spring grains are included in a crop rotation strategy, summer annual weeds are not favored.

Downloaded by UNIV MASSACHUSETTS AMHERST on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch020

Conclusions

It is important to recognize that many factors affected the $ynergy attributable to various pest management and crop production strategies. The resultant increases in crop yields reflect the effectiveness of the pest control resulting from the mixed-management strategies and the interaction of the environmental conditions. Generally, improved crop and pest management strategies will lessen the variability of the $ynergy. It is critical to select the appropriate pesticide, correct rate, and make a timely application to improve economic crop yield potential. Importantly, supplementing pesticides with alternative pest management strategies generally enhances $ynergy.

Typically, the greater the yield, the greater the $ynergy. However, if pest management costs are high, or if the pest effects on the crop are not severe, higher yields may not result in $ynergy. Growers must recognize the need for improved crop production and focus on specific field situations if $ynergy is to be a useful agronomic tool.

There are many factors, while not easily quantified, that should be considered when calculating $ynergy. These factors include the impact of escaped pests on harvesting efficiency, the effect of uncontrolled pests on future pest populations, and whether or not uncontrolled pests have an impact from a social perspective (esthetics). Given the high percentage of agricultural land that is rented, these factors are likely to be important considerations. Growers do make risk management decisions based on these factors, however, the actual economic benefit may not be quantifiable and thus this affects the calculation of $ynergy. Further, the impact of crop price is critically important for the calculation of $ynergy.

$ynergy depends on specific cropping situations and must consider the variability of risk. Generally, pest control will be improved with integrated management strategies, and thus will demonstrate $ynergy when compared to a single strategy. However, if environmental costs, future pest populations and other factors that are difficult or impossible to calculate could be factored, $ynergism would likely be more consistent and economically important. Regardless, the concept of crop-based $ynergy provides the grower with a more useful tool to assist in management decisions than synergy.

Downloaded by UNIV MASSACHUSETTS AMHERST on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch020

Literature Cited

1. Akobundu, I. O.; Sweet, R. D.; Duke, W. B. *Weed Sci.* **1975**, *23*, 20-25.
2. Akobundu, I. O.; Duke, W. B.; Sweet, R. D.; Minotti, P. L. *Weed Sci.* **1975**, *23*, 43-48.
3. Colby, S. R. *Weeds* **1967**, *15*, 20-22.

4. Green, J. M.; Bailey, S. P. In *Methods of Applying Herbicides*; McWhorter, C. G.; Gebhardt, M. R., Eds.; *Monogr. Ser. Weed Sci. Soc. Am.*; Weed Science Society of America, Champaign, IL, **1987**, *4*, pp 37-61.
5. Kudsk, P.; Thonke, K. E.; Streibig, J. C. *Weed Res.* **1987**, *27*, 425-429.
6. Morse, P. M. *Weed Sci.* **1978**, *26*, 58-71.
7. Streibig, J.C. *Ber. Fachg. Herbol.* **1983**, *24*, 183-193.
8. Green, J. M. *Weed Technol.* **1991**, *5*, 894-897.
9. Bullock, D.G. *Crit. Rev. Plant Sci.* **1992**, *11*, 309-326.
10. Duke, S. O.; Menn, J. J.; Plimmer, J. R. *Am. Chem. Soc. Symp.* **1993**, *524*, 1-13.
11. Gressel, J. *Am. Chem. Soc. Symp.* **1993**, *524*, 48-61.
12. Altenburger, R.; Bodeker, W.; Faust, M.; Grimme, L. H. *Ecotoxicol. Environ. Saf.* **1990**, *20*, 98-114.
13. Poch, G. Proc. 4th Inter. Conf. Comb. Effects Env. Fac. **1991**, 1-4.
14. Gressel, J. *Proc. 2nd Inter. Weed Cont. Cong.* **1996**, 1211-1221.
15. Hewlett, P.S. *Biometrics* **1969**, *25*, 477-487.
16. Tammes, P.M.L. *Neth. J. Plant Path.* **1964**, *70*, 73-80.
17. Byrd, J. D.; York, A. C. *Weed Technol.* **1988**, *2*, 433-436.
18. Waldrop, D. D.; Banks, P. A. *Weed Sci.* **1983**, *31*, 351-354.
19. Ahrens, W. H.; Ehr, R. J. *Weed Technol.* **1991**, *5*, 799-804.
20. Sorenson, V. M.; Meggitt, W. F.; Penner, D. *Weed Sci.* **1987**, *35*, 449-456.
21. Hagimoto, H.; Yoshikawa, H. *Weed Res.* **1972**, *12*, 21-30.
22. Dickerson, C. T., Jr.; Sweet, R. D. *Proc. North Cent. Weed Cont. Conf.* **1968**, *26*, 60.
23. Owen, M.D.K. *Proc. North Cent. Weed Cont. Conf.* **1985**, *40*, 89.
24. Smith, R. L.; Mohan, R. G.; Kollman, G. E. *Proc. North Cent. Weed Cont. Conf.* **1985**, *40*, 70-72.
25. Mekki, M.; Leroux, G. D. *Weed Technol.* **1994**, *8*, 436-440.
26. Blackshaw, R. C. *Weed Technol.* **1989**, *3*, 690-695.
27. Ezra, G.; Dekker, J. H.; Stephenson, G. R. *Weed Sci.* **1985**, *33*, 287-290.
28. Zorner, P. S. *Proc. North Cent. Weed Cont. Conf.* **1983**, *38*, 121.
29. Prosch, S. D.; Weber, J. B. *Proc. South. Weed Sci. Soc.* **1982**, *35*, 352-357.
30. Prosch, S. D.; Weber, J. B. *Proc. South. Weed Sci. Soc.* **1983**, *36*, 359-365.
31. Shaw, D. R.; Wesley, M. T. *Weed Technol.* **1993**, *7*, 693-698.
32. Flint, J. L.; Barrett, M. *Weed Sci.* **1989**, *37*, 12-18.
33. Agbakoba, C. S. O.; Goodin, J. F. *Weed Sci.* **1970**, *18*, 19-21.
34. Fedtke, C.; Strang, R. H. *Naturforschung.* **1990**, *45*, 565-567.
35. Minton, B. W.; Shaw, D. R.; Kurtz, M. E. *Weed Technol.* **1989**, *3*, 329-334.
36. Lich, J. M.; Renner, K. A.; Penner, D. *Weed Sci.* **1997**, *45*, 12-21.
37. Richer, D. L. *Pestic. Sci.* **1987**, *19*, 309-315.
38. Bodeker, W.; Altenburger, R.; Faust, M.; Grimme, L. H. *Nach Pflanzenschutzd.* **1990**, *42*, 70-78.
39. Poch, G. *Arch. Complex Eviron. Stud.* **1991**, *3*, 65-74.

Downloaded by UNIV MASSACHUSETTS AMHERST on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch020

40. Poch, G.; Reiffenstein, R. J.; Unkelbach, H. D. *Can. J. Pharmacol.* **1990**, *68*, 682-688.
41. Poch, G.; Dittrich, P.; Holzmann, S. *J. Pharmacol. Methods* **1990**, *24*, 311-325.
42. Streibig, J. C. *Weed Res.* **1986**, *26*, 207-214.
43. Gressel, J. *Rev. Weed Sci.* **1990**, *5*, 49-82.
44. Green, J. M. *Weed Technol.* **1989**, *3*, 217-226.
45. Nash, R. G. *Weed Sci.* **1981**, *29*, 147-155.
46. Swanton, C. J.; Weise, S. F. *Weed Technol.* **1991**, *5*, 657-663.
47. Teague, M. L.; Mapp, H. P.; Bernardo, D. J. *J. Prod. Ag.* **1995**, *8*, 405-415.
48. Bauer, T. A.; Mortensen, D. A. *Weed Technol.* **1992**, *6*, 228-235.
49. McWhorter, C. G.; Anderson, J. M. *Weed Sci.* **1981**, *29*, 245-252.
50. Jensen, P. K.; Kudsk, P. *Weed Res.* **1988**, *28*, 473-478.
51. Rao, S. R.; Harger, T. R. *Weed Sci.* **1981**, *29*, 208-212.
52. Klamroth, E. E.; Fedtke, C.; Kuhbauch, W. C. *Weed Sci.* **1989**, *37*, 517-520.
53. Wiles, L. J.; Wilkerson, G. G.; Gold, H. J. *Crop Protection* **1992**, *11*, 547-554.
54. Maxwell, B. D.; Colliver, C. T. *Proc. Brit. Crop Prot. Conf.* **1995**, 1069-1074.
55. Gunsolus, J. L.; Buhler, D. D. *J. Crop Prod.* **1999**, *2*, 167-187.
56. Collander, R. N. *Am. J. Ag. Econ.* **1989**, *71*, 996-1002.
57. Gunsolus, J. L. Amer. *J. Alt. Ag.* **1990**, *5*, 114-119.
58. Gressel, J.; Segel, L. E.; Ransom, J. K. *Intl. J. Pest Manage.* **1996**, *42*, 113-129.
59. DeFelice, M. S.; Brown, W. B.; Aldrich, R. J.; Sims, B. D.; Judy, D. T.; Guethle, D. R., *Weed Sci.* **1989**, *37*, 365-374.
60. Dieleman, J. A.; Mortensen, D. A. In *Integrated Weed and Soil Management*; Hatfield, J. L.; Buhler, D. D.; Stewart, B. A. Ed.; Ann Arbor Press: Ann Arbor, MI, **1998**; pp. 333-362.
61. Weaver, S. E. *Weed Technol.* 1991, 5, 674-679.
62. Hook, B. J.; Glenn, S. *Weed Sci.* **1984**, *32*, 691-696.
63. Genter, W. A. *Weeds* **1966**, *14*, 27-31.
64. Fawcett, R. S.; Slife, F. W. *Weed Sci.* **1975**, *23*, 419-424.
65. Einhelling, F. A. *Agron. J.* **1996**, *88*, 886-893.
66. Ditmarsen , S. C.; Sheppard, B. R.; Velovitch, J. J. *Proc. North Cent. Weed Sci. Soc.*, **1983**, *38*, 120.
67. Van Lieshout, L. A.; Loux, M. M. *Proc. North Cent. Weed Cont. Conf.* **1994**, *49*, 119.
68. Steckel, L. E.; DeFelice, M. S.; Sims, B. D. *Weed Sci.* **1990**, *38*, 541-545.
69. Gressel, J. *Brit. Crop Prot. Conf.* **1995**, 587-590.
70. Gardner, S. N.; Gressel, J.; Mangel, M. *Intl. J. Pest Manage.* **1998**, *44*, 161-180.
71. Minton, B. W.; Kurtz, M. E.; Shaw, D. R. *Weed Sci.* **1989**, *37*, 223-227.

Downloaded by UNIV MASSACHUSETTS AMHERST on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch020

72. Godley, J. L.; Kitchen, L. M. *Weed Sci.* **1986**, *34*, 936-941.
73. Hoverstad, T. R. *North Cent. Weed Sci. Soc. Res. Rep.*, **1997**, *54*, 412-413.
74. Fischer, D. W.; Harvey, R. G.; Doll, J. D. *North Cent. Weed Sci. Soc. Res. Rep.* **1997**, *54*, 360-361.
75. Mickelson, J. A.; Harvey, R. G.; Blaser, R.; Anthon, T. M. *North Cent. Weed Sci. Soc. Res. Rep.*, **1997**, *54*, 368-369.
76. Owen, M.D.K.; Lux, J. F.; Franzenburg, D. D.; Adam, K. W. *North Cent. Weed Sci. Soc. Res. Rep.* **1998**, *55*, 222-223.
77. Peterson, D. E.; Regehr, D. L. *North Cent. Weed Sci. Soc. Res. Rep.* **1997**, *54*, 223.
78. Corrigan, K. A.; Harvey, R. G.; Albright, J. W.; Anthon, T. M. *North Cent. Weed Sci. Soc. Res. Rep.* **1997**, *54*, 330-331.
79. Maxwell, D. J.; Wax, L. M.; Hart, S. E. *North Cent. Weed Sci. Soc. Res. Rep.* **1998**, *55*, 144-145.
80. Kalaher, C. J.; Stoller, E. W.; Maxwell, D. J. *North Cent. Weed Sci. Soc. Res. Rep.* **1998**, *55*, 170-171.
81. Regehr, D. L.; Peterson, D. E. *North Cent. Weed Sci. Soc. Res. Rep.* **1998**, *55*, 117-119.
82. Lovely, W. G.; Weber, C. R.; Staniforth, D. W. *Agron. J.* **1958**, *550*, 621-625.
83. Forcella, F.; Westgate, M. E.; Warnes, D. D. *Am. J. Alt. Agric.* **1992**, *7*, 161-167.
84. Hartzler, R. G.; Van Kooten, B. D.; Stoltenberg, D. E.; Hall, E. M.; Fawcett, R. S. *Weed Technol.* **1993**, *7*, 1001-1004.
85. VanGessel, M. J.; Schweizer, E. E.; Lybecker, D. W.; Westra, P. *Weed Technol.* **1995**, *9*, 754-760.
86. Mulder, T. A.; Doll, J. D. *Weed Technol.* **1993**, *7*, 382-389.
87. Mohler, C. L.; Frisch, J. C.; Mt. Pleasant, J. *Weed Technol.* **1997**, *11*, 123-131.
88. Mt. Pleasant, J.; Burt, F. R.; Frisch, J. C. *Weed Technol.* **1994**, *8*, 217-223.
89. Renner, K. A.; Woods, J. J. *J. Prod. Ag.* **1999**, *12*, 48-53.
90. Eadie, A. G.; Swanton, C. J.; Shaw, J. E.; Anderson, G. W. *Weed Technol.* **1992**, *6*, 535-542.
91. Buhler, D. D.; Gunsolus, J. L.; Ralston, D. F. *Weed Sci.* **1993**, *41*, 447-453.
92. Wolfe, M. S. *Proc. 2nd European Workshop on Integrated Control of Cereal Mildews* **1990**, *2*, 235-243.
93. Wolfe, M. S.; Brandle, U.; Koller, B.; Limpert, E.; McDermott, J. M.; Muller, K.; Schaffner, D. *Euphytica* **1992**, *63*, 125-139.
94. Vilich, V. *Crop Protection* **1993**, *12*, 373-379.
95. Hoffman, M. L.; Owen, M.D.K.; Buhler, D. D. *Agron. J.* **1998**, *90*, 793-799.

Downloaded by UNIV MASSACHUSETTS AMHERST on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch020

Chapter 21

Chloropropionic Acid Photoreduction in Solutions of Na_2S and Quinones

Kenley K. Ngim and Donald G. Crosby

Department of Environmental Toxicology, University of California, Davis, CA 95616

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch021

Photoreductive dechlorinations mediated by H_2S/HS^- are possible environmental mechanisms or may be applicable to the remediation of contaminated waters. Carfentrazone-chloropropionic acid {**I**; α,2-dichloro-5-[4-(difluoromethyl)-4,5-dihydro-3-methyl-5-oxo-1*H*-1,2,4-triazol-1-yl]-4-fluorobenzenepropanoic acid} is dechlorinated to its propionic acid {**II**; 2-chloro-5-[4-(difluoromethyl)-4,5-dihydro-3-methyl-5-oxo-1*H*-1,2,4-triazol-1-yl]-4-fluorobenzene-propanoic acid} in California flooded rice fields, and **I** and **II** are found in water and soil. While reductive dechlorinations are known to occur microbially, our purpose was to determine the reducing conditions promoting this reaction abiotically. Solutions of Na_2S alone and with quinones or natural rice field e^- carriers that were irradiated by UV were shown to dechlorinate **I**, yielding 1.6-28.4% of **II**; **II** was produced immediately, and maximum yields were obtained within 650 h. Dark reactivity was minor (0-2.5% of **II**). Only Na_2S with 1,4-benzoquinone produced a higher yield (28.4%) than Na_2S alone (19.8%), where the yield of **II** was directly proportional to Na_2S starting concentration and temperature. Minor dechlorination was demonstrated also in 4-chlorophenoxyacetic acid. Overall, Na_2S solution constituents were the primary photoreductants, while reduced quinones were more important in the photosensitized decomposition of **I**.

Photoreductions are used in organic synthesis and may be applicable as an environmental decomposition mechanism. Pesticide photoreductions in irradiated natural water solutions have been demonstrated extensively by our laboratory (*1,2,3*),

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch021

implying that reaction with reduced dissolved organic matter microenvironments occurs. Recent findings of mirex dechlorination in humic acid solutions irradiated by UV support this assumption (*4*). Also, findings of DDE dechlorination in irradiated sediment suspensions (*5*) extend the microenvironment theory to particulates. The difficulty with the above studies is that the photoreductants have not been characterized. Furthermore, it is unfortunate that virtually all studies using known reductants (*6,7,8,9*) were intended as synthetic methods. Consequently, organic solvent systems were used, making extrapolation to natural waters difficult.

Conversely, studies of "dark" reductions have been conducted in aqueous solution for certain types of chemicals (i.e., nitroaromatics, polychlorinated aliphatics), and enrichment with known reductants prevents any uncertainty over e^- donating species. In fact, the pathway of nitro reduction has been determined (*10*), and all such abiotic reductions are believed to involve similar sequential e^- transfer to an δ^+ atom, followed by protonation. Such reactions are believed to occur via redox coupling, analogous to biological e^- transfer in photosynthesis or in oxidative metabolism.

In the environment, the bulk e^- donors for abiotic reductions include sulfides (i.e., H_2S) and Fe(II) (*11*), and these are often used in *in vitro* studies. Certain functionalities may serve as e^- carriers, including quinones and organometal complexes, due to inherent interchangeability of e^- acquisition and release; these may be constituents of aquatic organic matter (i.e., dissolved, colloidal, particulate) or soil surfaces. While redox coupling with other oxidized mediators is possible, e^- transfer to a reducible group on a pollutant molecule normally terminates this chain. Reduction under such schemes has been demonstrated for polyhaloaliphatics (*12,13*) and substituted nitrobenzenes (*14,15*).

The notion that carfentrazone-chloropropionic acid {**I**; α,2-dichloro-5-[4-(difluoromethyl)-4,5-dihydro-3-methyl-5-oxo-1*H*-1,2,4-triazol-1-yl]-4-fluorobenzenepropanoic acid} may be reduced in a similar manner was prompted by observations of rapid hydrolysis of carfentrazone-ethyl herbicide {ethyl α,2-dichloro-5-[4-(difluoromethyl)-4,5-dihydro-3-methyl-5-oxo-1*H*-1,2,4-triazol-1-yl]-4-fluorobenzenepropanoate} to **I** and identification of the corresponding propionic acid {**II**; 2-chloro-5-[4-(difluoromethyl)-4,5-dihydro-3-methyl-5-oxo-1*H*-1,2,4-triazol-1-yl]-4-fluorobenzenepropanoic acid} as the major degradation product in California rice field water and soil (*16*) (Figure 1). However, the polarity imparted by a single Cl is considerably less than that of typically used chemicals, so we anticipated that the dark reaction would occur far more slowly, or maybe not at all. Our objective was to characterize reducing conditions that would facilitate such an unfavorable reaction, so we used UV radiation as a driving force. This study would allow a better understanding of the environmental fate of carfentrazone-ethyl, while utilizing known reductants and solutions relevant to natural waters.

Materials & Methods

Standard Photoreduction Parameters

Triplicate UV exposures were conducted in glass-stoppered Pyrex glass reactors (*17*), each outfitted with a single F40/350BL lamp (Sylvania-GTE). Solution temperature (35°C) was maintained by a plastic tubing jacket connected to a contant temperature circulator. Dark trials were conducted in Teflon-capped, amber glass bottles (1 L) maintained in a water bath. Solutions were flushed with N_2 for 30 min (30 mL/min) to purge O_2; dark control headspace was purged after each opening. UV trials were run for 600-800 h or >3 dissipation half-lives of **I**, while dark controls lasted as long as corresponding UV trials.

Model solutions consisted of 0.1 M phosphate buffer (pH 7) containing Na_2S (26 mM), e^- carrier, and **I** (1.4 μM). Quinone e^- carriers (0.13 mM) included benzoquinone {1,4-benzoquinone}, juglone {5-hydroxy-1,4-naphthoquinone}, or lawsone {2-hydroxy-1,4-naphthoquinone}. A 20% rice straw extract in 0.1 M phosphate buffer and unbuffered rice field water were used for comparison. The straw extract was made by incubating 150 g of dried rice straw (Colusa County, CA) in 3.75 L distilled deionized water for 6 months, at 24°C in Teflon-capped, amber glass jugs. Field water and straw extracts were filtered (0.22 μm) prior to use. Controls consisted of dark trials for the above and UV/dark trials of Na_2S alone, reduced quinones alone, e^- carrier alone, and phosphate buffered solutions of **I**. Reduced quinones included hydroquinone and hydrojuglone; the latter is not commercially available and was synthesized according to a published method (*18*).

Samples (25 mL) were acidified (pH 2-2.5) with 10% H_2SO_4 (1.0 mL) and passed through 3 cc, 500 mg, C-18 solid-phase extraction (SPE) cartridges (Varian, Harbor City, CA). Ether eluates (2 mL) were methylated with diazomethane in ether, evaporated to near dryness under N_2, reconstituted in hexane (2 mL), and adjusted to 1 mL by further evaporation and volumetric glassware. Analysis of **I**, **II**, and other products (i.e., benzoic, cinnamic acids) was by GC with a thermionic specific detector (Varian, Walnut Creek, CA) fitted with a 15 m x 0.32 mm i.d. column containing a 0.25 μm film of DB-1701 (J&W, Folsom, CA). Reduction product identity was verified by GC with a mass-selective detector (Hewlett Packard, Wilmington, DE) operated in scan mode and fitted with a 30 m x 0.25 mm i.d. column containing a 0.25 μm film of DB-5 (J&W). Mass spectra from authentic standards were used for confirmation.

Supplemental Studies

Changes from the above standard parameter trials were necessary to confirm the photoreducing activity of Na_2S with benzoquinone and better understand the

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch021

mechanism of photoreductive dechlorination. To validate photoredox coupling between **I** and reduced quinoids, the Na_2S starting concentration was lowered from 26 mM to 1.3 mM, while benzoquinone remained constant (0.13 mM). Next, since Na_2S appeared to be the primary reductant, trials without e^- carriers were run as follows: concentration (1.3 mM and 91 mM), pH (5 and 9), temperature (24°C and 50°C) in the dark; findings contrasted with those from Na_2S standard condition controls (26 mM, pH 7, 35°C).

4-Chlorophenoxyacetic acid (0.13 mM) was investigated as a model monochloroaromatic compound to determine how universal the photoreduction mechanism might be. This experiment was conducted with Na_2S (no e^- carrier) under standard conditions. Samples (50 mL) were analyzed by acidification with 20% H_2SO_4 and C-18 SPE. Cartridges were thoroughly dried prior to elution with methanol (2 mL). Analytes (phenoxyacetic acid, 4-chlorophenol, and phenol) were detected using reverse-phase HPLC with UV/Vis detection (Isco, Lincoln, NE) at 237 nm, with acetonitrile:H_2O:acetic acid (50:49:1) as the mobile phase. Separation was achieved with a 250 mm x 4.6 mm i.d. Alltima column packed with 5 μm C-18 particles (Alltech, Deerfield, IL).

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch021

Chloropropionic Acid Dissipation

Dissipation of **I** under UV irradiation was dictated by degree of photosensitized degradation, photoreduction, and a dark reaction. Half-life ($t_{1/2}$) (Table I) was calculated from pseudo-first-order kinetics. The least stability was observed with juglone and lawsone ($t_{1/2}$ 3.58 h and 8.42 h, respectively), indicating that combinations with Na_2S act as potent photosensitizing agents in the decomposition of **I** and possibly of the reduction product. Indeed, the photosensitizing activity of quinoids is known (*19*), and that of hydroquinone on methoxychlor solutions was demonstrated (*7*). Rice straw extract and benzoquinone ($t_{1/2}$ 88.2 h and 260 h, respectively) were less effective. The rice straw extract was intended to simulate anaerobic straw decomposition in a rice field. Natural organic matter contains polyphenolic groups, so quinoid functions may be present in the rice straw extract. Also, as the extract was incubated under nonsterile conditions, various microbial metabolites may be present, as well. As the composition of the rice straw extract was not characterized, however, the dissipation of **I** cannot be attributed conclusively to photosensitized degradation with reduced quinones. Rice field water and Na_2S alone ($t_{1/2}$ 528 h and 467 h, respectively) produced no effects. The field water may contain insufficient organic matter to enhance sensitized photolysis. The dissipation of **I** is illustrated for Na_2S alone and with juglone (Figure 2).

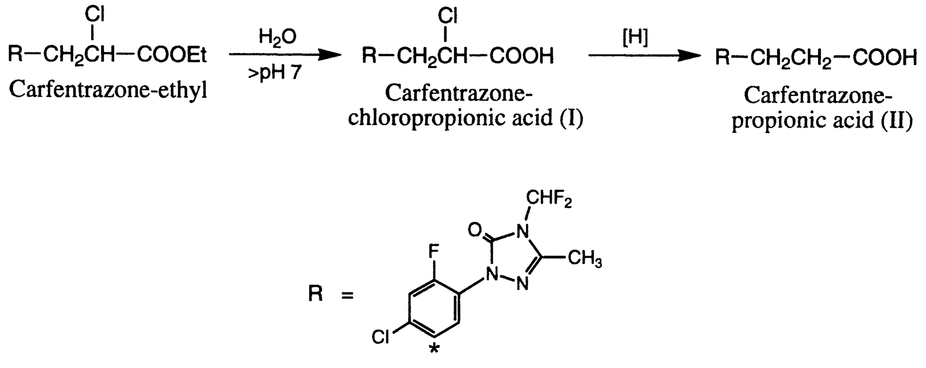

Cl
R—CH2CH—COOEt
Carfentrazone-ethyl
H2O
>pH 7
Cl
R—CH2CH—COOH
Carfentrazone-chloropropionic acid (I)
[H]
R—CH2CH2—COOH
Carfentrazone-propionic acid (II)
R =
CHF2
O
N
N
N
CH3
F
Cl
*

Figure 1. Primary carfentrazone-ethyl decomposition route in California rice field water and soil. Base hydrolysis to carfentrazone-chloropropionic acid (I) precedes reduction to corresponding propionic acid (II).

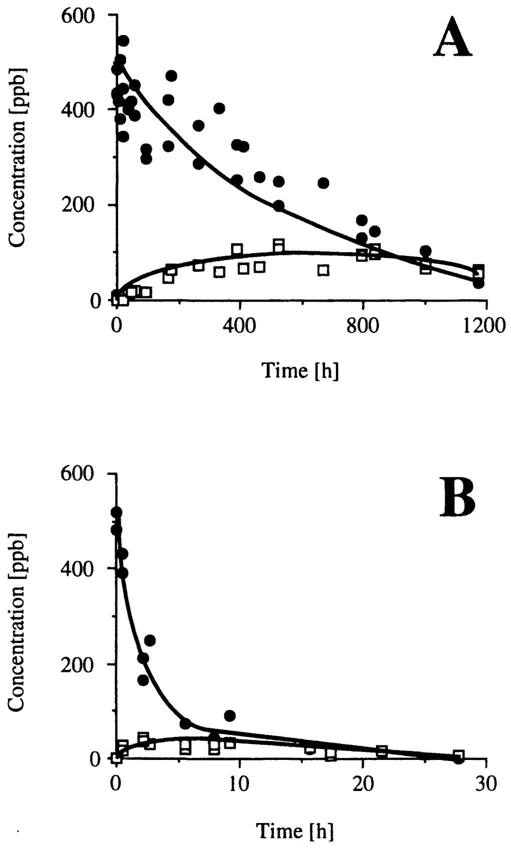


Figure 2. Dissipation of carfentrazone-chloropropionic acid (squares) to corresponding propionic acid (circles) in solutions of Na_2S (A) and Na_2S with juglone (B) irradiated by UV light.

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch021

Table I. Chloropropionic Acid Stability and Yield of Its Propionic Acid Metabolite in Various Na_2S Solutions Irradiated by UV Light

Trial[a]	*$t_{1/2}$ (h)*	*Yield of Compound II (% of Initial I)*
Na_2S only	467 +/- 88	19.8 +/- 6.9
Benzoquinone	260 +/- 16	28.4 +/- 10.2
Juglone	3.58 +/- 0.53	8.48 +/- 1.63
Lawsone	8.42 +/- 3.37	1.64 +/- 0.51
Rice Straw Solution (20%)	88.2 +/- 10.9	18.4 +/- 5.9
Rice Field Water (unbuffered)	528 +/- 108	14.8 +/- 2.9

[a] *n* = 3 for each

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch021

I was generally more stable in the corresponding dark controls for this experiment ($t_{1/2}$ 360 h or greater). Juglone and lawsone provided stability, as both were run only as long as their UV trials (50-60 h) and **I** did not dissipate in this short period. In the presence of rice straw, benzoquinone, and rice field water **I** was less stable ($t_{1/2}$ 360 h, 435 h, and 568 h, respectively). The exception was Na_2S ($t_{1/2}$ 465 h), which produced comparable UV dissipation kinetics, suggesting that a dark reaction occurs. Since e^- donation from H_2S/HS^- is sequential, free-radical sulfur species may be involved. The dissipation in e^- carrier controls (no Na_2S) ranged from giving $t_{1/2}$ values comparable to corresponding Na_2S, trials to being stable; dark trials also were stable.

Stability of **I** was greater in hydrojuglone and hydroquinone UV/dark controls (20% degraded after 53 h and $t_{1/2}$ 337 h, respectively) than corresponding Na_2S trials with the quinone. The differences might indicate that hydroquinones had been oxidized more rapidly in the absence of Na_2S to less photoactive quinones or that Na_2S and quinones work in tandem as reactants. Also, it is known that mercaptoquinones are formed in solutions of Na_2S and quinones via Michael addition (*12*). Thus, these may be acting as even more potent photosensitizing agents than are the corresponding hydroquinones.

Dissipation of **I** in benzoquinone with 1.3 mM Na_2S was far greater than corresponding 26 mM trials ($t_{1/2}$ 10.4 h vs. 260 h, respectively), indicating that H_2S/HS^- disrupts photosensitized decomposition by reduced quinones. This agrees with the more rapid decomposition in direct photolysis trials ($t_{1/2}$ 358 h). Stability of **I** in Na_2S trials at 1.3 mM and 91 mM (<15% lost after 800 h and $t_{1/2}$ 122.5 h, respectively) was consistent with 26 mM standard conditions ($t_{1/2}$ 467 h). The similarity of UV and dark controls further supports the occurrence of a dark reaction. Stability of **I** at 24°C and 50°C (36% lost after 800 h and $t_{1/2}$ 337 h, respectively) was compatible with standard (35°C) Na_2S dark controls ($t_{1/2}$ 465 h). Increasing temperature likely accelerates the dark reaction. Dissipation of **I** was enhanced significantly at pH 9 ($t_{1/2}$ 281 h) and inhibited at pH 5 (only 30% lost after 700 h). Dissipation in corresponding dark controls was similar at pH 5 (20% lost), while significant dark reaction inhibition occurred at pH 9 (30% lost). Volatility loss of H_2S during preparation of pH 5 solutions, confirmed electrochemically using an S^{2-} probe, apparently diminished reactants responsible for the dark reaction. Poor correlation of UV and dark findings at pH 9 indicates that photoactive sulfide compounds are formed at basic pH.

The persistence of 4-chlorophenoxyacetic acid was comparatively higher for Na_2S and direct photolysis in pH 7 buffer trials (1,921 h and 745 h, respectively). Again, the inhibitory effect of Na_2S on direct photolysis was demonstrated. Both dark trials were stable, indicating that 4-chlorophenoxyacetic acid is stable to the dark reaction that degraded **I**.

Photoreduction Product Yields

Reduction product yields (Table I) were dictated by photosensitized degradation and, possibly, photoredox coupling efficiency. Lawsone and juglone produced the least **II** (1.64% and 8.48%, respectively). Based on the rapid photosensitized degradation discussed previously of these trials, it is possible that **I** reacts more rapidly by this reaction than by photoreduction, thereby lowering yields of **II**. Also, the photosensitized degradation of **II** could similarly diminish photoreduction yields. Similarly, trials in which **I** dissipated the slowest should produce the most **II**. Indeed, the highest yields were achieved with Na_2S alone and with benzoquinone (19.8% and 28.4%, respectively), which were among the most stable ($t_{1/2}$ 467 h and 260 h, respectively). Inconsistencies with field water (14.8%), which provided the most stability ($t_{1/2}$ 529 h), and rice straw extract (16.8%), which was among the least stable ($t_{1/2}$ 88.2 h), are likely due to differences in photoredox coupling efficiency. Production of **II** over time occurs with Na_2S alone and with juglone (Figure 2).

Photoredox coupling with reduced quinones was established unequivocally for benzoquinone trials with lower initial Na_2S (1.3 mM). A 2.4% yield was achieved with benzoquinone, while quantities detected with 1.3 mM Na_2S alone were below the limit of quantitation (LOQ) and the dark controls yielded no reduction product. These findings substantiate those of the standard parameter trials, in which photoreduction with Na_2S and benzoquinone exceeded those with Na_2S alone, and suggest that UV also enhances redox coupling with reduced quinones.

Yields of reduction product were directly proportional to the Na_2S starting concentration and temperature, but diminished as pH was lowered. Yields at 1.3 mM and 91 mM (<LOQ and 50.7%, respectively) were consistent with those of standard (26 mM) Na_2S trials (19.8%) and confirmed Na_2S as a photoreductant. While variable between different temperatures, maximum yield for any given run did correlate with temperature (8.7% and 2.5% at 50°C and 35°C, respectively; <LOQ at 24°C). Although redox coupling occurs in the dark and is enhanced by heat, UV proved a superior driving force in the reductive dechlorination. Diminished yields with declining hydrogen ion is apparent between pH 7 (19.8%) and pH 9 (14.7%), probably due to less effective protonation of the anionic intermediate formed prior to **II** at basic pH. At pH 5, Na_2S loss as volatile H_2S (mentioned above) decreased reductant concentrations, resulting in the undetectability of **II**.

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch021

Photoreductive dechlorination on an aromatic ring was confirmed with 4-chlorophenoxyacetic acid. While quantitation of phenoxyacetic acid or phenol, two possible reduction products, was difficult by reverse-phase HPLC, methylation followed by GC-MS successfully demonstrated the reactivity. While phenoxyacetic acid was readily detected in Na_2S trials and confirmed by authentic standards, no reduction product was evident from either direct photolysis trials or dark trials. That only traces were detected in this case, compared to above trials, is not surprising since e^- donation to a delocalized aromatic ring is more difficult, regardless of degree of δ^+.

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch021

Photoredox Coupling in Summary

Chloropropionic acid photoreduction by solutions of Na_2S alone and with quinones or rice extract constituents can be explained on the basis of redox coupling (Figure 3). Direct coupling can occur with Na_2S solution constituents such as H_2S/HS^- and polysulfides formed by reaction of S^o (from H_2S/HS^- oxidation) with remaining H_2S/HS^- (*13,20*). The importance of polysulfides as reductants was demonstrated in a recent study of hexachloroethane reduction in the dark (*13*). Based on the high yield of **II** achieved with Na_2S relative to other e^- carriers except benzoquinone, it appears that Na_2S solution constituents serve as the primary reductants. The reactivity of 4-chlorophenoxyacetic acid confirms that reduction of monochloroaromatic compounds also occurs under this scheme.

One must also consider the effect of Na_2S redox coupling on the e^- carriers themselves. The reduced forms re-oxidize with e^- transfer to a reducible substrate, and, due to potentially more intimate near-space association, could even enhance reduction over that of the bulk reductant alone. Although photosensitized decomposition is generally promoted by quinoid chemicals, thereby diminishing the yield of reduction products, the use of benzoquinone confirmed that redox coupling with reduced quinones was occurring and that they could be important contributors to photoreduction. Hydroquinones and mercaptohydroquinones may also contribute to the observed formation of reduction products.

Conclusions

Monochlorinated aliphatic and aromatic compounds are often more persistent in the environment than their polychlorinated counterparts because they are more difficult to reduce and, consequently, more slowly mineralized. The photodechlorination process reported here would require that natural reductants, such as H_2S/HS^- and organic matter be present in sufficient quantities in sunlit waters to allow such reactions to occur. Certainly, flooded rice paddies are candidates, because they contain saturated soil environments rich in organic matter, and are irradiated with intense solar UV. Adequate H_2S/HS^- levels in the water could render this reaction an important pesticide decomposition mechanism. Also, application as a remediation

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch021

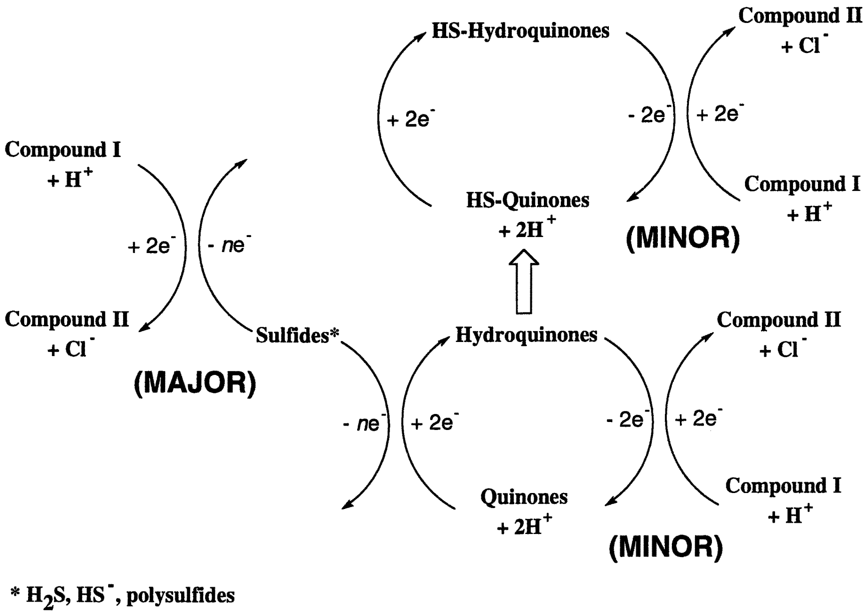

HS-Hydroquinones
Compound II
+ Cl⁻
+ 2e⁻
- 2e⁻
+ 2e⁻
Compound I
+ H⁺
HS-Quinones
+ 2H⁺
Compound I
+ H⁺
(MINOR)
+ 2e⁻
- ne⁻
Compound II
+ Cl⁻
Sulfides*
Hydroquinones
Compound II
+ Cl⁻
(MAJOR)
- ne⁻
+ 2e⁻
- 2e⁻
+ 2e⁻
Quinones
+ 2H⁺
Compound I
+ H⁺
(MINOR)
* H₂S, HS⁻, polysulfides

Figure 3. Possible photoredox coupling in the photoreduction of carfentrazone-chloropropionic acid (I) to corresponding propionic acid (II) in the presence of Na_2S and quinones. Sulfides, hydroquinones, and mercaptoquinones could be the reductants.

strategy seems plausible. Work is currently in progress, using 2-chloropropionic acid as a simpler carfentrazone analog, to determine the effect of the aryl triazolinone backbone on reactivity, and to further confirm photoreductive dechlorination.

References

1. Nakagawa, M.; Crosby, D.G. *J. Agric. Food Chem.* **1974**, *22*, 849-853.
2. Crosby, D.G.; Wong, A.S. *J. Agric. Food Chem.* **1973**, *21*, 1052-1054.
3. Moilanen, K.W.; Crosby, D.G. *J. Agric. Food Chem.* **1972**, *20*, 950-953.
4. Burns, S.E.; Hassett, J.P.; Rossi, M.V. *Environ. Sci. Technol.* **1996**, *30*, 2934-2941.
5. Miller, G.C.; Zepp, R.G. *Environ. Sci. Technol.* **1979**, *13*, 860-863.
6. Hsu, T-L.C.; Chang, I-J.; Ward, D.L.; Nocera, D.G. *Inorg. Chem.* **1994**, *33*, 2932-2937.
7. Chaudhary, S.K.; Mitchell, R.H.; West, P.R. *Chemosphere* **1985**, *14*, 27-40.
8. Shimizu, N.; Watanabe, K.; Tsuno, Y. *Bull. Chem. Soc. Jpn.* **1984**, *57*, 885-886.
9. Bunce, N.J.; Bergsma, J.P.; Bergsma, M.D.; De Graaf, W.; Kumar, Y.; Ravanal, L. *J. Org. Chem.* **1980**, *45*, 3708-3713.
10. March, J. *Advanced Organic Chemistry*, 3rd ed.; John Wiley & Sons: New York, NY, 1985; pp 1103-1104.
11. Dunnivant, F.M.; Schwarzenbach, R.P.; Macalady, D.L. *Environ. Sci. Technol.* **1992**, *26*, 2133-2141.
12. Perlinger, J.A.; Buschmann, J.; Angst, W.; Schwarzenbach, R.P. *Environ. Sci. Technol.* **1998**, *32*, 2431-2437.
13. Perlinger, J.A.; Angst, W.; Schwarzenbach, R.P. *Environ. Sci. Technol.* **1996**, *30*, 3408-3417.
14. Schwarzenbach, R.P.; Stierli, R.; Lanz, K.; Zeyer, J. *Environ. Sci. Technol.* **1990**, *24*, 1566-1574.
15. Klausen, J.; Trober, S.P.; Haderlein, S.B.; Schwarzenbach, R.P. *Environ. Sci. Technol.* **1995**, *29*, 2396-2404.
16. Ngim, K.K.; Crosby, D.G., unpublished results.
17. Crosby, D.G.; Tang, C. *J. Agric. Food Chem.* **1969**, *32*, 728-733.
18. Fieser, L.F.; Williamson, K.L. *Organic Experiments*; D.C. Heath and Company: Lexington, MA, 1975; p 335.
19. Schwarzenbach, R.P.; Gschwend, P.M.; Imboden, D.M. *Environmental Organic Chemistry*; John Wiley & Sons: New York, NY, 1993; p 445.
20. Roberts, A.L.; Sanborn, P.N.; Gschwend, P.M. *Environ. Sci. Technol.* **1992**, *26*, 2263-3374.

Downloaded by COLUMBIA UNIV on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ch021

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ix001

Author Index

Alber, T., 194
Barkovskii, Andrei L., 283
Boger, Peter, 253
Bollag, Jean-Marc, 182
Corio-Costet, M.-F., 166
Crosby, Donald G., 397
Crowley, D. E., 333
Dec, Jerzy, 182
Feng, Paul C. C., 129
Forlani, Giuseppe, 145
Gressel, Jonathan, 376
Hall, J. C., 2, 89, 194
Hatzios, K. K., 218
Hertkorn, N., 119
Hoagland, R. E., 2, 58, 194
Kafarski, Pawel, 145
Lange, B. M., 119
Lee, Hung, 194
Lejczak, Barbara, 145
Locke, Martin A., 194
Luepromchai, E., 333
May, R. G., 119
Mena, Frank L., 310
Mougin, C. P., 166
Ngim, Kenley, K., 397
Owen, Michael, 376
Ramsey, Ryan J. L., 310
Ruff, Thomas G., 129
Sadowsky, Michael J., 268
Sandermann, Jr., H., 119
Schmidt, Burkhard, 40
Schocken, Mark J., 30
Shaner, Dale L., 353
Singer, A., 333
Sommer, Alfred, 253
Stephenson, Gerald R., 310
Tecle, Berhane, 353
Trevors, Jack T., 194
Vuilleumier, Stephane, 240
Wackett, Lawrence P., 268
Werck-Reichhart, D., 166
Wickenden, J. Sue, 89
Yau, Kerrm Y. F., 89
Zablotowicz, R. M., 2, 58, 194

Subject Index

A

Accelerated biodegradation, pesticide fate, 14–15
Acylanilides
  amide hydrolysis in plants, 64–65
  structural examples, 66*f*
  structure-activity study, 72–73
Acylation, conjugation reaction in fungi, 107
Agricultural production, pesticides transforming, 2–3
Alkaloids
  structurally diverse biosynthetic compounds in nature, 333–334
  See also Cometabolism of xenobiotics
Altered metabolism, approach for making crop tolerant to glyphosate, 370
Altered translocation, action of safeners, 323
Amide hydrolysis
  microorganisms, 67–73
  plants, 64–65, 67
  See also Microorganism enzymes; Plant enzymes
Amino acid conjugation
  callus versus whole plant, 96–97
  conjugates of 2,4-D in soybean callus, 96
  conjugation in pea, 96*f*
  herbicidal activity, 97
  metabolism of 2,4-D amino acid conjugates, 97
  prevention of morphogenesis, 97–98
  types of metabolites, 95–96
2-Aminoethanephosphonic acid, catabolism, 146–147
Analytical techniques, methodology, 18–19
Animal bioavailability, plant bound pesticidal residues, 124–125
Animal metabolism
  metolachlor, 36, 38*f*
  microbial synthesis approach for predicting metabolites, 31–32
Antagonism
  competitive, theory of safener action, 324
  mechanisms of herbicide safeners in plants, 328*t*
Antagonists, examples of herbicide/pesticide interactions in plants, 11, 12*t*
Antibody techniques, methodology, 19–21
Antidotes. *See* Safeners
Antisera production
  method and Western blotting, 256–257
  See also Recombinant isoforms of glutathione S-transferase (GST) from corn
Arabidopsis thaliana, bacterial glutathione S-transferase (GST), 243, 244*f*, 245*t*
Aromatic compounds
  oxidative metabolism in plants, 6
  ring cleavage in mineralization, 6
Aromatic nitroreduction
  partial, 207–208
  See also Microbial reductive pathways
Arsenic compounds, methylation by Challenger mechanism, 110
Aryl acylamidase
  activity on five substrates in cell-free extracts from ten bacterial strains, 69*t*
  colorimetric method for measuring, 70–71
  competitive inhibition of activity, 73
  interaction of chemical structure and microbial, activity, 72
  microbial species producing, and related hydrolytic enzymes, 68*t*
Aryloxyphenoxypropionates, possible herbicide/safener combinations and uses, 312*f*

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ix002

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ix002

Ascorbic acid oxidation
  degradation of carbamate insecticide, matacil, 36–37
  identification of metabolites, 37, 38*f*
Assays
  antibody technology, 19–21
  examples of ELISA assays for common pesticides, 20*t*
Atrazine
  ability to degrade unrelated bacteria, 278
  atrazine-degrading bacteria, 270, 272
  degradation, 269–270
  degradation by various hydrolases from *Pseudomonas* sp., 188*f*
  degradation pathway, 187
  enhanced microbial degradation under marsh systems, 303
  genetics of degradation, 272
  herbicide uses and action, 269
  metabolic profiling, 47–49
  metabolic rates, 47*t*
  resistance from detoxification by glutathione conjugation, 226
  structure, 274*f*
  See also Plant cell suspension cultures
Atrazine degradation genetics
  atrazine chlorohydrolase (AtzA), 273, 275
  atzA gene ideal for engineering bacteria and plants to metabolize atrazine, 273, 275
  atzB gene second in catabolic pathway, 275
  atzC gene third in catabolic pathway, 275
  complementation approach to isolate and characterize gene regions of *Pseudomonas* sp. strain ADP encoding, 273
  metabolic pathway by which *Pseudomonas* strain ADP mineralizing atrazine to CO2 and ammonia, 274*f*
  overall metabolic logic, 276
  possible mechanisms for atzA, B, and C transfer among bacteria, 276
  studies of AtzA properties, 275

B

Bacteria
  atrazine-degrading, 270, 272
  some s-triazine degrading bacteria, substrates, and degradation products, 271*t*
Bacterial conjugation reactions
  glutathione, 112
  methylation, 108, 110–111
  methylation of divalent mercury, 110
  O-methylation conjugation environmentally important, 110–111
  nitrosation, 111–112
  transformation of arsenic compounds, 110
Bacterial glutathione S-transferases
  applications, 249–250
  carbon–carbon double bond isomerization by bacterial GSTs, 246*f*
  characterization of biodegradative pathway for degradation of isoprene and chlorinated ethenes, 247–248
  comparison of aligned structures of, from *Proteus mirabilis*, alachlor-active GST from *Arabidopsis thaliana*, and model of human GSTT, 244*f*
  dependence of enzyme-catalyzed carbon–carbon double bond isomerization reactions on glutathione, 248
  dichloromethane dehalogenation by bacterial GSTs, 246*f*
  epoxide ring-opening by bacterial GSTs, 246*f*
  GST-associated metabolism in bacteria, 248–249
  GSTs of known function, 245–248
  occurrence of GST-dependent pathways for degradation of xenobiotics, 240–241
  percent structural versus sequence similarity between GSTs of known X-ray structure, 245*t*
  preferential uptake of GST substrate by bacteria, 249
  reductive dehalogenation of chlorinated hydroquinones by bacterial GSTs, 246*f*
  sequence and structural similarity in, 241–245
  sequence similarity of, to genomic sets of GST genes, 242*t*
  toxicity of GST substrates, 249
  See also Glutathione S-transferases (GSTs)
Benoxacor, inducing sulfate assimilation, 318
Bentazon
  coupling reaction in soil, 108
  formation of O-glucoside of, in rice, 93*f*
  humification of, catalyzed by laccase of *Polyporus pinsitus*, 109*f*

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ix002

Bialaphos
  anti-fungal activity, 80
  chemical structure, 79*f*
  genetic engineering of crops for, resistance, 78–80
Biochemical conjugation. *See* Conjugation
Biodegradation
  accelerated, 14–15
  research on plant signal compounds, 334–335
Biological implications, synergy, 383–384
Bioremediation
  advantages of enzymatic treatment, 183
  application of microorganisms, 183
  decontamination of water and soil using various oxidoreductases, 189
  degradation of atrazine by various hydrolases from *Pseudomonas* sp., 188*f*
  enzymes showing ability to degrade xenobiotics, 187
  enzymes showing potential for pesticide hydrolysis, 186*t*
  enzyme stabilization for maximized life-times, 189–190
  enzymes tested for, 184, 187
  flavonoids, 341–343
  fungal cytochrome P-450s, 178–179
  hydrolysis of phenyl carbamate by esterase from *Pseudomonas* sp., 185*f*
  hydrolysis rates for organophosphate pesticides, 185*t*
  induction of broad specificity enzymes a factor of concern, 335
  pesticide fate, 15
  phytochemicals of interest for, 337–343
  plant terpenoids, 337–340
  representative plant compounds of interest for, 339*f*
  salicylic acid, 340–341
  using plant materials containing peroxidase activity, 190–191
Biotechnology approaches. *See* Crop selectivity
Bound pesticidal residues in crop plants
  animal bioavailability, 124–125
  definition of bound xenobiotic residues of plant and animal origin, 120
  differentiation between xenobiotic and natural labeling, 121
  distribution of radioactivity among plant cell wall fractions, 122*t*
  endogenous binding partners, 121–122
  fractionation of plant cell wall components, 121*t*
  incorporation rates of radiolabeled pesticides into nonextractable residue fractions of whole plants and plant cell suspension cultures, 119, 120*t*
  linkage types in bound residues, 122–123
  microbial bioavailability, 124
  mild acid hydrolysis under simulated stomach conditions, 126*f*
  plant metabolic studies with radioactively labeled pesticides documenting, 119
  proposed linkage types for chloroaniline/pectin, chloroaniline/lignin, and phenoxyacetic acid/carbohydrate polymers, 123*f*
  toxicology, 125–126
Broad-spectrum herbicides, approaches to discover new, 354
Bromoxynil
  cotton varieties with bromoxynil tolerant trait, 369
  description, 367
  designing herbicide tolerance, 367, 369
  metabolism, 368*f*
  nitrile hydrolysis in microorganisms, 77
  nitrile hydrolysis in plants, 77

C

Camphor
  biodegradation in *Mycobacterium rhodochrous*, 338
  structure, 339*f*
  See also Cometabolism of xenobiotics
Captan, inhibition of esterases, 64
Carbamate-hydrolase, hydrolysis in microorganisms, 75
Carbamates
  degradation of matacil by ascorbic acid oxidation system, 36–37
  hydrolysis in microorganisms, 74–75
  hydrolysis in plants, 74
  identification of metabolites, 38*f*
  possible herbicide/safener combinations and uses, 312*f*
  propanil hydrolysis inhibition, 73
Carbamothioates, possible herbicide/safener combinations and uses, 312*f*
Carbon–carbon double bond isomerization, catalysis by bacterial glutathione S-transferase (GST), 246*f*, 248

Carbon nutrition, distinction between plant and microbial metabolism, 4–5
Carbon–phosphorus bond. *See* Organophosphonates
Carfentrazone-ethyl herbicide. *See* Chloropropionic acid photoreduction
Chemical technology, core strategy for controlling pests, 3
Chloramben
  hydrolysis by esterases, 62
  metabolism, 359, 363*f*
  metabolism in plants, 99
  metabolism in tolerant and susceptible species, 99*f*
Chlorimuron-ethyl, safener enhancing metabolism, 322
Chloroacetamide herbicides
  amide hydrolysis, 71–72
  possible herbicide/safener combinations and uses, 312*f*
Chloroacetanilide herbicides
  activity of glutathione S-transferases towards, 221
  safeners enhancing glutathione conjugation, 229
Chloroaniline/lignin conjugates. *See* Lignin/chloroaniline conjugates
4-Chlorobiphenyl, enzymatic transformations, 187
1-Chloro-2,4-dinitrobenzene (CDNB), assaying plant glutathione S-transferase (GST) activity, 220
Chloropropionic acid photoreduction
  chloropropionic acid dissipation, 400, 402–403
  chloropropionic acid stability and yield of its propionic acid metabolite in various UV-light irradiated Na_2S solutions, 402*t*
  dissipation of carfentrazone-chloropropionic acid to corresponding propionic acid in solutions of sodium sulfide(Na_2S) and Na_2S with juglone irradiated by UV light, 401*f*
  photoredox coupling, 404
  photoreduction product yields, 403–404
  possible photoredox coupling in photoreduction of carfentrazone-chloropropionic acid to corresponding propionic acid in presence of Na_2S and quinones, 405*f*
  standard photoreduction parameters, 399
  supplemental study methods, 399–400
Chlorothalonil, nitrile hydrolysis in plants, 77
Chlorotoluron
  difference spectra of wheat cell microsomes, 172*f*
  inhibition of metabolism by P-450 inhibitors in wheat and Persian speedwell, 173*t*
  metabolism in cell cultures, 170
  metabolism in microsomal preparations, 171
  metabolism in plants, 170
  role of cytochrome P-450 in metabolism and selectivity, 169–170
Ciliatine, catabolism, 146–147
Clomazone herbicide, microbial metabolism in rats, soybeans, and soil, 33, 34*f*
Cloning. *See* Recombinant isoforms of glutathione S-transferase (GST) from corn
Cometabolic transformation, reductive dehalogenation, 288, 290
Cometabolism
  broader definition, 334
  definition, 3–4, 90
  survival advantage, 90–91
  See also Conjugation
Cometabolism of xenobiotics
  biodegradation of monoterpenes, 338
  biodegradation pathway for camphor in *Mycobacterium rhodochrous*, 338
  chlorinated metabolites undergoing dehalogenation prior to degradation pathway, 335–336
  community analysis of soil bacteria enriched by selected phytochemicals, 347*f*
  correspondence analysis of bacteria communities showing relative similarities and differences in community structures after enrichment, 348*f*
  degradation of flavonoids, 342–343
  enrichment of xenobiotic degraders on terpenes, 346
  flavonoids, 341–343
  monooxygenase enzymes in, 336
  PCB (polychlorinated biphenyl) degradation dependence on removal of inhibitory metabolites from chlorobenzoates, 344
  photochemicals of interest for bioremediation, 337–343
  plant beneficial effects for enhancing

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ix002

    biodegradation of chlorobenzoates, 344–345
  plant induction of PCB cometabolism, 343–346
  plant terpenoids, 337–340
  polycyclic aromatic hydrocarbon (PAH) degradation in rhizosphere, 343
  representative plant compounds of interest for bioremediation, 339*f*
  research on terpenes, 338, 340
  rhizosphere effect, 336
  rhizosphere effects on biodegradation of xenobiotics, 336
  salicylate inducible toluene monooxygenase enzyme, 341
  salicylic acid, 340–341
  terpene enhanced PCB degradation, 345–346
  terpenes only inducing gram-positive bacteria, 345–346
  xenobiotic compounds degradable by, 335
Competitive antagonism, theory of safener action, 324
Computer-aided molecular modeling, safener/herbicide combinations, 324
Conjugation
  cometabolism, 90–91
  definition, 90
  extension of definition, 113–114
  fates of conjugates in plants and microorganisms, 91
  reactions in living organisms, 113
  reaction types, 112
  similarities between reactions in plants and microorganisms, 90, 113
  transformation in microbes, 113
  xenobiotic conjugation reactions recognized or presumed to occur in plants and microorganisms, 91*t*
Conjugation reactions in microorganisms
  acetylation of 4-bromoaniline by fungal cultures of *Talaromyces wortmanni* and *Fusarium oxysporum*, 107*f*
  acylation, 107
  bacterial conjugation reactions, 108, 110–112
  glutathione conjugation, 112
  humification of bentazon catalyzed by laccase of *Polyporus pinsitus*, 109*f*
  metabolism of oxadiazon by *Fusarium solani*, 106*f*
  methyl conjugation, transformation of oxadiazon by *Fusarium solani*, 106
  methylation in bacteria, 108, 110–111
  methylation in fungi, 104–106
  O-methylation of pentachlorophenol by fungal cultures of *Trichoderma virgatum*, 105*f*
  nitrosation in bacteria, 111–112
  reactions in fungi, 103–108
  significance of xenobiotic conjugation by fungi, 108
  transformation of arsenic by gram-positive and -negative bacteria, 110*f*
  xylosation of 2,4-D by *Dichomitus squalens*, 104*f*
  xylosylation in fungi, 103–104
Conjugation reactions in plants
  amino acid conjugates of 2,4-D in soybean callus, 96
  amino acid conjugation, 95–98
  amino acid conjugation in pea, 96*f*
  callus versus whole plant in amino acid conjugation, 96–97
  case study of metribuzin, 101–102
  chloramben metabolism, 99
  detoxification of pesticides by, 92
  differential conjugation affecting tolerance of plant, 100–101
  enzymatic conjugation of flurodifen to glutathione in pea, 95*f*
  formation of glucose ester of 2,4-D in wheat, 93*f*
  formation of O-glucoside of bentazon in rice, 93*f*
  glucose conjugation, 92–93
  glutathione conjugation, 94–95
  herbicidal activity of amino acid conjugates, 97
  immobilization of pesticides, 98
  imparting selectivity, 99–101
  insoluble residues, 98
  malonic acid conjugation, 94
  metabolism of 2,4-D amino acid conjugates, 97
  metabolism of chloramben in tolerant and susceptible species, 99*f*
  metabolism of metribuzin in tomato, 102
  picloram metabolism, 100–101
  prevention of morphogenesis, 97–98
  proposed metabolic scheme for picloram in leafy spurge, 100*f*
  selectivity differences for metribuzin among soybean cultivars, 101–102

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ix002

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ix002

  three phases of herbicide metabolism, 92
  types of metabolites in amino acid conjugation, 95–96
  uridine diphosphate glucose (UDPG) as donor for formation of glucose esters, 93
  vacuolar compartmentalization, 98
Corn. *See* Maize; Recombinant isoforms of glutathione S-transferase (GST) from corn
Cotton, varieties containing bromoxynil tolerant trait, 369
Crop rotations
  advantages, 377
  $ynergy, 392
  $ynergy from, 392
  See also Synergy
Crop selectivity
  biotechnology approaches, 366–370
  bromoxynil, 367, 369
  (2,4-dichlorophenyl)acetic acid (2,4-D), 366–367
  differential rates and routes to metabolism, 354
  glufosinate, 369–370
  glyphosate, 370
  plant breeding, 366
Crops
  genetic engineering for bialaphos/glufosinate resistance, 78–80
  herbicide resistant, 10
Cyanazine, nitrile hydrolysis in plants, 77
Cyclohexadiones, possible herbicide/safener combinations and uses, 312*f*
Cyprodinil
  metabolite production, 34*f*
  microbial transformation study predicting environmental fate, 32
Cytochrome P-450
  first cloning of plant P-450 gene, 168
  functions, 169
  influence of safeners, 316
  knowledge concerning regulation, 168–169
  properties of plant and microbial, 167–169
  safeners inducing activity of P-450 monooxygenases, 315
  species specific, 316–317
  superfamily of genes encoding, 168
Cytochrome P-450, fungal
  evidence of P-450s in filamentous fungi, 178
  involvement in lindane transformation, 178
  mass balance analysis of ^{14}C in soil cultures after 9-week incubation, 179*t*
  soil bioremediation, 179
  tools for bioremediation, 178–179
Cytochrome P-450, plant
  combined effects of fenpropimorph and agrochemicals on sterol profiles, 175
  difference spectra of wheat cell microsomes, 172*f*
  effect of treatment of seedlings with agrochemicals on microsomal P-450 content, 176*t*
  effects of fenpropimorph with agrochemicals on wheat P-450 content, 175–176
  experimental for studying effects of fenpropimorph on sterol profiles, 174
  GC–EI–MS spectrum of oxygenated metabolite from fenpropimorph by wheat microsomal preparations, 177*f*
  inhibition of chlorotoluron metabolism by P-450 inhibitors in wheat and Persian speedwell, 173*t*
  involvement of P-450 in fenpropimorph metabolism, 176
  metabolism of chlorotoluron in cell cultures, 170
  metabolism of chlorotoluron in microsomal preparations, 171
  metabolism of chlorotoluron in plants, 170
  P-450 genes in phenylurea metabolism, 173
  role in fungicide metabolism in host plant, 174–176
  role in herbicide cross-resistance in weeds, 173–174
  role in herbicide metabolism and selectivity, 169–173
  sterol distribution in wheat seedlings after 10-day treatment with fenpropimorph, 175*t*
Cytological techniques, methodology, 21

D

De-esterification. *See* Engineering plant tolerance to pyridine herbicides
Defense compounds, enzymes in synthesis, 228

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ix002

Defense genes, plant glutathione transferases, 220
Degradation, role of phosphatases and sulfatases in pesticide, 77
Dehalogenation, dichloromethane, catalysis by bacterial glutathione S-transferase (GST), 245, 246*f*
Dehalogenation reactions
  enzymes inducing by, 285
  mechanisms, 284–285, 286*t*
  molecular structures of naturally occurring halogenated aromatic compounds, 287*f*
  See also Reductive dehalogenation
Dehydrohalogenation, mechanism, 285, 286*t*
Denitrifiers, capable of reductive dehalogenation, 296
Designing herbicide tolerance
  biotechnology approaches, 366–370
  bromoxynil, 367, 369
  crop selectivity through plant breeding and biotechnology, 366–370
  crop tolerance of 5-substituted imidazolinone analogs, 356*t*
  (2,4-dichlorophenyl)acetic acid, 366–367
  discovery and optimization of imidazolinone herbicides, 354–359
  glufosinate, 369–370
  glyphosate, 370
  metabolism of sulfonylureas and other herbicides, 359, 364
  plant breeding for herbicide tolerance, 366
  reasons for unpredictability of metabolism of herbicides, 364, 366
  structures of lead imidazolinone herbicides and optimized analog, 355*f*
  See also Imidazolinone herbicides; Sulfonylurea herbicides
Dethylsimazine, degrading bacteria, substrates, and products, 271*t*
Diazinon, enzymatic hydrolysis rates, 185*t*
Dichlobenil, nitrile hydrolysis in plants, 77
Dichlormid
  inducing sulfate assimilation, 318
  safener for carbamothioate herbicides, 310–311
Dichloromethane dehalogenation, catalysis by bacterial glutathione S-transferase (GST), 245, 246*f*
2-4-Dichlorophenol, bioremediation using plant materials containing peroxidase activity, 190–191
(2,4-Dichlorophenyl)acetic acid (2,4-D)
  designing herbicide tolerance, 366–367
  metabolism by *Alcaligenes eutrophus*, 368*f*
  transformation by dioxygenase, 187
Diclofop-methyl
  ester hydrolysis in microorganisms, 63
  ester hydrolysis in plants, 60, 62
Dinitroaniline herbicides
  aromatic nitroreduction, 204, 206
  nitroaromatic transformations in plants, 210–213
Dinitrophenols
  aromatic nitroreduction, 204, 206
  bacterial hydrogenation reactions, 208–209
Diphenylether herbicides
  aromatic nitroreduction, 202–204
  interest in modifying crop tolerance, 213
Discovery, herbicide, target validation and target identification, 18
Dithiopyr
  custom synthesis, 130
  structure, 130*f*
  time course of hydrolysis of pyridine herbicides by porcine liver esterases, 136*f*
  time course of oxidation of pyridine herbicides by NADPH-fortified rat liver microsomes, 135*f*
  See also Pyridine herbicides
Diuron, insoluble residues, 98

E

Economics. *See* Synergy
Endosulfan, role for sulfatase in fungal metabolism, 77
Engineering plant tolerance to pyridine herbicides
  achieving tolerance via metabolic deactivation mechanism, 133–134
  amino acid alignment of cloned endoplasmic reticulum (ER)-*rle3* with published rle1, 138*f*
  assay methods for thiazopyr tolerance, 133
  assays for thiazopyr tolerance in tobacco and tomato, 142
  comparison of *N*-terminal amino acid

- residues of purified pyridine esterase (*rle3*) and published esterase isozymes (*rle1* and *rle2*) from rabbit liver, 137*t*
- confirmation of thiazopyr as target of deactivation, 134
- correlating tolerance to expression of *rle3* esterase, 143
- detection of plant *rle3* esterase activity in vitro and in vivo, 141–142
- detection of *rle3* esterase in tissue extracts from tomato and tobacco lines by Western blot analysis, 140*f*
- HPLC profiles from UV and ^{14}C detection showing in vivo hydrolysis of MON 14300 to seed in control and two lines of transgenic tomato seedlings, 141*f*
- identification of deactivation pathway, 134–136
- insect expression of *rle1* and *rle3* cDNAs, 139
- known families of enzymes catalyzing de-esterification, 135
- materials and methods, 130–133
- method for expression of *rle1* and *rle3* cDNAs in insect cells and in plants, 133
- other interesting observations, 143
- PCR cloning of rabbit liver esterases cDNAs, 132
- PCR cloning of rabbit liver esterases (*rle1* and *rle3*), 137–139
- plant transformation of *rle3* cDNA, 140
- potential pathways of pyridine de-esterification via oxidation or hydrolysis, 134*f*
- procedures for in vitro and in vivo assays for esterase activity, 131
- purification method of pyridine-esterases from porcine and rabbit liver, 131–132
- purification of rabbit liver pyridine-esterase, 136–137
- thiazopyr dose titration under preplant incorporation in control and transgenic tomato seeds, 143*f*
- time course of hydrolysis of pyridine herbicides by porcine liver esterases, 136*f*
- time course of oxidation of pyridine herbicides by NADPH-fortified rat liver microsomes, 135*f*
- in vitro oxidation method by rat liver microsomes, 131

Environment
- accelerated biodegradation, 14–15
- bioremediation, 15
- effects on pesticide metabolism in plants, 13
- pesticide fate, 13–16
- phytoremediation, 16
- synergy and environmental considerations, 383

Environmental fate
- degradation of carbamate insecticide, matacil, by ascorbic acid oxidation system, 36–37, 38*f*
- microbial metabolism of clomazone herbicide in rats, soybeans, and soil, 33, 34*f*
- microbial production of metabolites of tetrazolinone herbicide, 33
- microbial synthesis approach predicting metabolites in soil, animals, and plants, 31–32
- microbial transformations by fungus *Absidia pseudocylindraspora*, 33, 36
- plant metabolism by in vitro methods, 37
- predicting cyprodinil metabolism by microbial approach, 32, 34*f*
- structures of metabolites and possible bioconversion pathway for *A. pseudocylindraspora*, 35*f*
- *See also* In vitro methods

Enzymes
- applications, 183
- degrading xenobiotics, 187
- high cost of isolating, 183
- immobilization for stabilizing, 189–190
- safener enhancing, 322–323
- testing for bioremediation of organophosphate pesticides, 184, 187

Epoxide ring-opening, catalysis by bacterial glutathione S-transferase (GST), 246*f*, 247–248

Escherichia coli, bacterial glutathione S-transferase (GST), 243, 245

Ethenes degradation, chlorinated, catalysis by bacterial glutathione S-transferase (GST), 246*f*, 247–248

Expression, enzymes in metabolic pathway, 7

Extraction and purification methods, chemical analytical technique, 18–19

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ix002

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ix002

F

Fast protein liquid chromatography (FPLC), chemical analytical technique, 19
Fenclorim, safener, activity of glutathione S-transferases towards, 222
Fenitrothion, enzymatic hydrolysis rates, 185*t*
Fenoxaprop-ethyl
  ester hydrolysis in microorganisms, 63
  ester hydrolysis in plants, 60, 62
Fenpropimorph
  combined effects of, and agrochemicals on sterol profiles, 175
  effect of treatment of seedlings with agrochemicals on microsomal P-450 content, 176*t*
  effects with agrochemicals on wheat cytochrome P-450 content, 175–176
  experimental for studying effects on sterol profiles, 174
  GC–EI–MS spectrum of oxygenated metabolite from fenpropimorph by wheat microsomal preparations, 177*f*
  involvement of P-450 in metabolism, 176
  sterol biosynthesis inhibitor, 174
  sterol distribution in wheat seedlings after 10-day treatments with, 175*t*
Flavin monooxygenases
  oxidative biotransformations of nitroaromatic compounds, 196–198
  possible pathways for 2,4-dinitrophenol degradation, 200*f*
Flavonoids
  degradation, 342–343
  description and function, 341–342
  root colonizing pseudomonads influencing production, 342
  See also Cometabolism of xenobiotics
Flumetsulam
  detoxification, 364
  detoxified by hydroxylation of aromatic methyl, 364
  differential routes of metabolism, 365*f*
Fluorodifen, activity of glutathione S-transferases towards, 222–223
Folpet, inhibition of esterases, 64
Fungi
  acylation, 107
  metabolism of oxadiazon by *Fusarium solani*, 106*f*
  methylation reactions, 104–106
  transformation of oxadiazon, 106
  xylose conjugation, 103–104
  See also Conjugation reactions in microorganisms
Fungicide metabolism. *See* Fenpropimorph
Fungicides, examples of pesticide mineralization by pure cultures of bacteria and fungi, 5*t*

G

Gel electrophoresis techniques, molecular biology technique, 17
Gene cloning, molecular biology technique, 16–17
Gene–enzyme system, glutathione transferase (GST)
  GST genes in plants, 224–225
  GST isozymes in plants, 221–224
  system of maize, 223*t*
Gene expression
  regulation of glutathione S-transferase, 231, 233
  reporter gene (β-glucuronidase) in *Arabidopsis thaliana*, 326
  safener-responsive genes, 326
  safeners as inducers, 325–326
Gene libraries, use of, molecular biology technique, 16–17
Genetic engineering
  crops for bialaphos/glufosinate resistance, 78–80
  See also Engineering plant tolerance to pyridine herbicides; Recombinant isoforms of glutathione S-transferase (GST) from corn
Genetics
  atrazine and s-triazine degradation, 272
  atrazine degradation in *Pseudomonas* sp. strain ADP, 272–276
  See also Atrazine degradation genetics
Genomics
  sequence similarity of bacterial glutathione S-transferases (GSTs) to genomic sets of GST genes, 242*t*
  study of gene structure and function, 17–18
  target validation and target identification, 18

Glucose conjugation
  formation of glucose ester of 2,4-D in wheat, 93*f*
  formation of O-glucoside of bentazon in rice, 93*f*
  reactions in plants, 92–93
Glucose esters, vacuolar compartmentalization, 98
Glufosinate
  anti-fungal activity, 80
  bar gene, 369–370
  biodegradation, 149
  designing herbicide tolerance, 369–370
  genetic engineering of crops for, resistance, 78–80
  metabolic profiling, 50–52
  metabolic rates after 48 h incubation, 51*t*
  metabolism, 371*f*
  microorganisms producing bialaphos protecting selves, 369
  plant metabolic pathways, 51*f*
  See also Plant cell suspension cultures
Glutathione (GSH)
  concentration in plants, 218
  conjugation of xenobiotics, 94
  conjugation reaction in bacteria, 112
  conjugation reaction in plants, 94–95
  cysteine availability limiting production, 318
  enzymatic conjugation of flurodifen to glutathione in pea, 95*f*
  GSH conjugation, oxidative stress, and herbicide metabolism in plants, 317
  multiple functions of GSH in higher plants, 219
  non-enzymatic conjugation with GSH in plants, 318–319
  pumps, 233
  role of GSH conjugation in herbicide metabolism, 225–228
  safeners affecting aspects of GSH mediated detoxification, 321–322
  safeners enhancing GSH formation, 317–318
Glutathione peroxidase
  activities of recombinant corn GST isoforms, 261–262
  activity assays, 257
  See also Recombinant isoforms of glutathione S-transferase (GST) from corn
Glutathione S-transferases (GSTs)
  assay activity using 1-chloro-2,4-dinitrobenzene (CDNB), 220
  catalytic mechanisms of plant GSTs detoxifying herbicides, 226, 228
  catalyzing conjugation with wide range of substrates, 253
  chemical structures of herbicide safeners known to enhance GST activity, 230*f*
  chemical structures of herbicides known to conjugate with glutathione (GSH) in plants, 227*f*
  chemical structures of herbicide synergist tridiphane and of GS-tridiphane conjugate, 232*f*
  classification, 220
  classifications of cytosolic plant GSTs in crops, 254–255
  concentration of GSH in plants, 218
  enzyme catalyzed reactions of xenobiotics in plants, 314*f*
  fate of GSH conjugates in plants, 233
  functions of plant, 219–220
  grain sorghum GST isozymes, 222
  GST gene–enzyme system, 221–225
  GST gene–enzyme system of maize, 223*t*
  GST genes in plants, 224–225
  GST isozymes in plants, 221–224
  identification and study of plant GSTs, 254
  legume crops, GST isozymes, 223–224
  maize GST isozymes, 221–222
  multifunctional enzymes, 219
  Northern blot analysis of total RNA isolated from roots of rice seedlings after safener treatment, 232*f*
  regulation of glutathione-mediated reactions, 228–233
  regulation of GST gene expression, 231, 233
  rice GST isozymes, 222
  safener-enhanced GST activity, 319–320
  safeners and GSH-mediated reactions, 228–229
  synergists and GSH-mediated reactions, 229, 231
  wheat GST isozymes, 222
  See also Bacterial glutathione S-transferases; Recombinant isoforms of glutathione S-transferase (GST) from corn
Glyphosate
  biodegradation, 149–151
  designing herbicide tolerance, 370
  metabolism, 371*f*
  mineralization, 149–150

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ix002

Grain sorghum, glutathione S-transferase (GST) isozymes in plants, 222
Growth-linked metabolism, nonselective priming of degrader organisms, 334

H

Halogenated compounds
  ubiquity in environment, 285
  See also Dehalogenation reactions; Reductive dehalogenation
Halo-respiration
  bacterial cultures with capability, 292*t*
  halogenated compounds serving as terminal acceptors in anaerobic environments, 290, 293
  mechanism of energy conservation through, 293
  presence of microorganisms in rhizosphere, 297
  specific enzymes, 293
Herbicide cross-resistance in weeds, role of plant cytochrome P-450s, 173–174
Herbicide/cultivation interactions
  economics of $ynergy from mechanical strategies, 390–392
  risk and mechanical strategies, 390
  $ynergy from, 389–392
Herbicide discovery, target validation and target identification, 18
Herbicide metabolism
  differential effects of safeners on, in crops and weeds, 324–325
  glutathione conjugation, oxidative stress, and, in plants, 317
  role of glutathione conjugation, 225–228
Herbicide resistance
  managing in weeds, 9–10
  mechanisms, 9*t*
  multiple, 9–10
Herbicides
  approaches to discover broad-spectrum herbicides, 354
  catalytic mechanisms of plant glutathione S-transferases (GSTs) detoxifying, 226, 228
  chemical structures of herbicides conjugating with glutathione, 226, 227*f*
  detoxification by glutathione conjugation, 226
  examples of pesticide mineralization by pure cultures of bacteria and fungi, 5*t*
  phase II metabolism, 317
  phase I metabolism, 315
  possible herbicide/safener combinations and uses, 312*f*
  reasons for unpredictability of metabolism of, 364, 366
  resistant crops, 10
  See also Designing herbicide tolerance; Safeners
Heterotrophic plant cell suspension cultures
  attributes, 42
  metabolism of xenobiotics by, 42–43
  See also Plant cell suspension cultures
Hydration mechanism, 286*t*
Hydrogenation reactions, bacterial, dinitrophenols, 208–209
Hydrolytic dehalogenation, mechanism, 285, 286*t*
Hydrolytic enzymes
  amide hydrolysis in microorganisms, 67–73
  amide hydrolysis in plants, 64–65, 67
  carbamate hydrolysis in microorganisms, 74–75
  carbamate hydrolysis in plants, 74
  cellular compartmentalization, 59
  ester hydrolysis in microorganisms, 63–64
  ester hydrolysis in plants, 59–62
  genetic engineering of crops for bialaphos/glufosinate resistance, 78–80
  hydrolysis of organophosphate insecticides by microorganisms, 76
  hydrolysis of organophosphate insecticides by plants, 75–76
  inhibition of esterases in plants and microorganisms, 64
  inhibition of plant and microbial amidases, 73
  nitrile hydrolysis in microorganisms, 77
  nitrile hydrolysis in plants, 77
  pesticide degradation, 59
  proposed mechanism for catalytic activity of esterase superfamily, 66*f*
  role of phosphatases and sulfatases in pesticide degradation, 77
  See also Microorganism enzymes; Microorganisms; Plant enzymes; Plants
Hydroquinones, reductive dehalogenation of chlorinated, catalysis by bacterial glutathione S-transferase (GST), 246*f*, 247

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ix002

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ix002

Hydroxylation, cytochrome P-450 and influence of safeners, 316

I

Imidazolinone herbicides
  crop sensitivity depending on rates and routes of metabolism, 356
  crop tolerance to 5-substituted imidazolinone analogs, 356*t*
  detoxification by peanut, soybean, and maize, 356, 359
  differential route of metabolism of imazamethylabenz in wheat and wild oats, 359, 360*f*
  differential routes of metabolism of 5-methyl, ethyl, and methoxymethyl imidazolinone analogs in maize, peanut, and soybean plants, 357*f*
  empirical approach for discovery, 354
  metabolic pathways of imazapic in soybean seedlings, 358*f*
  possible herbicide/safener combinations and uses, 312*f*
  structures of lead, and optimized analog, 355*f*
Immobilization, enzyme stabilization, 189–190
Immobilization of pesticides
  insoluble residues, 98
  vacuolar compartmentalization, 98
Insecticides
  biodegradation of organophosphonate, 155–157
  examples of pesticide mineralization by pure cultures of bacteria and fungi, 5*t*
Insoluble residues, immobilization of pesticides, 98
Integrated pest management
  importance of pesticide interactions, 376
  See also Synergy
Interactions. *See* Synergy
Intramolecular substitution, mechanism, 286*t*
In vitro methods
  benefits of microbial approach, 32
  definition, 31
  degradation of carbamate insecticide using ascorbic acid oxidation system, 36–37, 38*f*
  identification of metabolites involving single, double, and triple hydroxylations at different sites in metolachlor molecule, 36, 38*f*
  microbial production of metabolites from tetrazolinone herbicide, 33
  microbial synthesis approach predicting metabolites in soil, animals, and plants, 31–32
  microbial transformations by filamentous fungus *Absidia pseudocylindraspora*, 33, 36
  plant metabolism, 37
  reasons to use, 31
  role in facilitating metabolite identification in animals, 36
  structures of metabolites and possible bioconversion pathway for *A. pseudocylindraspora*, 35*f*
  transformation of metolachlor, 36, 38*f*
Ipridione, amide hydrolysis, 73
Isoprene degradation, catalysis by bacterial glutathione S-transferase (GST), 246*f*, 247–248

L

Legal synergy, 380–381
Legume crops, glutathione S-transferase (GST) isozymes in plants, 223–224
Lignin/chloroaniline conjugates
  animal bioavailability, 124–125
  linkage, 122–123
  microbial bioavailability, 124
Lindane
  involvement of cytochrome P-450s in transformation, 178
  soil bioremediation, 179

M

Maize
  discovery of safener binding protein, 326–327
  glutathione S-transferase (GST) isozymes in plants, 221–222
  GST gene–enzyme system, 223*t*
  GST genes, 224–225
  synthetic gene encoding GST, 225
  See also Glutathione S-transferases (GSTs)
Malonic acid conjugation, reaction in plants, 94
Mass spectrometry (MS), identification and structural analysis, 19

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ix002

Matacil, carbamate insecticide, degradation by ascorbic acid oxidation system, 36–37, 38*f*
Mathematical synergy, 381, 383
Matrix-assisted laser desorption ionization–time of flight (MALDI–TOF) mass spectrometry, technique, 19
Melamine, degrading bacteria, substrates, and products, 271*t*
Mercury, divalent, methylation to methyl mercury, 110
Metabolic alteration. *See* Designing herbicide tolerance
Metabolic pathways
  integration, 7
  phase I for herbicides, 315
  phase II for herbicides, 317
Metabolic profiling
  atrazine, 47–49
  glufosinate, 50–52
  metabolic rates of glufosinate after 48 h incubation, 51*t*
  metabolic rates of parathion, atrazine, and pyrene, 47*t*
  observations using plant cell suspension cultures, 45–46
  parathion, 46–47
  parathion using plant cell suspension cultures, 47*f*
  pesticides and xenobiotics using plant cell suspension cultures, 52*t*
  plant metabolic pathways of glufosinate, 51*f*
  pyrene, 49–50
  standardized plant cell culture metabolism test, 43–45
  See also Plant cell suspension cultures
Metabolic rates
  glufosinate after 48 h incubation, 51*t*
  parathion, atrazine, and pyrene, 47*t*
  pesticides in wheat and soybean cell suspension cultures, 45*t*
  See also Metabolic profiling; Plant cell suspension cultures
Methylation
  conjugation reactions in bacteria, 108, 110–111
  conjugation reactions in fungi, 104–106
  metabolism of oxadiazon by *Fusarium solani*, 106*f*
  O-methylation of pentachlorophenol by fungal cultures of *Trichoderma virgatum*, 105*f*
Metobromuron, acylation by fungi, 107
Metolachlor
  activity of glutathione S-transferases towards, 222
  animal metabolism, 36
  metabolites with single, double, and triple hydroxylations, 38*f*
Metribuzin
  conjugation case study, 101–102
  description and uses, 101
  metabolism in tomato, 102
  plant breeding for herbicide tolerance, 366
  selectivity differences among soybean cultivars, 101–102
Microbial bioavailability, plant bound pesticidal residues, 124
Microbial degradation, possible mechanisms, 284
Microbial metabolism
  fundamental differences between plant and, 4*t*
  influence of plants on, of xenobiotics, 284
Microbial oxidative pathways
  flavin monooxygenases, 196–198
  metabolism of 2,4-dinitrophenol initiated by flavin monooxygenase, monooxygenase, or dioxygenase, 200*f*
  monooxygenases for removal of nitro groups, 195–196
  toluene dioxygenase for nitrotoluene metabolism, 199–201
  See also Nitroaromatic pesticides
Microbial reductive pathways
  aromatic nitroreduction of herbicides, 202–207
  bacterial hydrogenation reactions, 208–209
  biochemical transformations versus chemical nitroreduction processes, 201–202
  Clostridium bifermentans degrading dinoseb under anoxic conditions, 210
  examples of reductive and partial reductive transformation of nitroaromatic compounds by bacteria and fungi, 202*t*
  GC–MS spectrum of trifluralin and its amino-derivative metabolite, 205*f*
  microaerophilic metabolism of dinoseb by resting cell suspensions, 206*t*
  nitroreduction of diphenylether herbicides, 202–203

partial aromatic nitroreduction, 207–208
partial reductive nitroreductases, 201
potential for aromatic nitroreduction of dinitroaniline and dinitrophenol herbicides, 204
potential of fungi to transform or mineralize 2,4,6-trinitrotoluene (TNT), 210
reducing ^{14}C-trifluralin to mono amino derivative under microaerophilic conditions, 203–204
transformation of trifluralin and dinoseb under aerobic and microaerophilic conditions, 203–204
transformations of trifluralin resulting from initial aromatic nitroreduction, 207*f*
See also Nitroaromatic pesticides; Reductive dehalogenation

Microbial synthesis approach
  ability to overload cultures, 32–33
  benefits, 32
  predicting metabolites in soil, animals, and plants, 31–32
  screening cultures in fungicide cyprodinil metabolism, 32, 34*f*
  studying metabolism of herbicide clomazone in rats, soybeans, and soil, 33, 34*f*

Microorganism enzymes
  amide hydrolysis, 67–73
  aryl acylamidase activity on five substrates in cell-free extracts from ten bacterial strains, 69*t*
  aryl acylamidases, 67
  ^{14}C-propanil metabolism by cell suspensions of soil, water, and rhizosphere bacteria, 71*t*
  carbamate hydrolysis, 74–75
  chloroacetamide herbicides, 71–72
  colorimetric method for measuring aryl acylamidase activity in soil, 70–71
  ester hydrolysis, 63–64
  fenoxaprop-ethyl and diclofop-methyl degradation in soils, 63
  hydrolysis of organophosphate insecticides, 76
  inhibition of esterases, 64
  inhibition of microbial amidases, 73
  interaction of chemical structure and microbial aryl acylamidase activity, 72
  nitrile hydrolysis, 77
  propanil hydrolysis major mechanism for dissipation in soil, 68, 70
  proposed mechanism for catalytic activity of esterase superfamily, 66*f*
  recovery of propanil-hydrolyzing isolates from Mississippi Delta rice soil and flood water, 70*t*
  selected microbial species producing aryl acylamidases and related hydrolytic enzymes, 68*t*
  source of aryl acylamidases, 72
  structure-activity study evaluating acylanilide herbicide, 72–73
  See also Pesticide metabolism in plants and microorganisms

Microorganisms
  aerobic metabolism under anoxic conditions using alternate terminal electron acceptors, 6–7
  See also Conjugation reactions in microorganisms

Microwave-assisted extraction (MAE), chemical analytical technique, 18

Mineralization
  description, 5
  desiring enzymatic action for complete, 183
  examples of pesticide mineralization by pure cultures of bacteria and fungi, 5*t*
  facilitating complete xenobiotic, 6
  glyphosate, 149–150
  microbial adaptation for, of s-triazine herbicides, 277
  ring cleavage of aromatic pesticides, 6

Molecular biology techniques, pesticide chemistry, 16–17

Molecular modeling, safener/herbicide combinations, 324

MON 2300 and MON 14300
  custom synthesis, 130
  structure of pyridine herbicide, 130*f*
  time course of hydrolysis of pyridine herbicides by porcine liver esterases, 136*f*
  time course of oxidation of pyridine herbicides by NADPH-fortified rat liver microsomes, 135*f*
  See also Pyridine herbicides

Monooxygenases
  oxidative removal of nitro groups from nitrophenols, 195–196

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ix002

  safeners inducing cytochrome P-450, 315
  study in relation to cometabolism, 336
Morphogenesis, prevention, 97–98
Multiple pest control strategies, cost of using in concert, 378

N

Naphthalene, biodegradation, 187
1,8-Naphthalic anhydride
  first commercial safener, 310
  See also Safeners
Nerve agents, degradation, 156–157
Nicosulfuron
  detoxification by hydroxylation of pyrimidine ring, 359
  effect of structural substituents and application rates of nicosulfuron analogs on maize injury, 364*t*
  metabolic pathway in maize, 362*f*
Nitrile hydrolysis, plants and microorganisms, 77
Nitroaromatic pesticides
  aromatic nitroreductases of aerobic and anaerobic bacteria, 201
  aromatic nitroreduction in fungi, 206
  aromatic nitroreduction of herbicides, 202–207
  bacterial hydrogenation reactions, 208–209
  bacteria metabolizing nitrobenzene using partial reduction process, 201
  biochemical transformations versus chemical nitroreduction processes, 201–202
  comparative metabolism of, by cultures of *Sphingomonas* sp. strain UG30 and *Escherichia coli* BL21 (pBX2) suspensions expressing pentachlorophenol (PCP)-flavin monooxygenase (pcpB) gene, 197*t*
  degradation of nitrobenzene by *Pseudomonas pseudoalcaligenes* strain JS52, 208
  dinoseb degradation under anoxic conditions by *Clostridium bifermentans*, 210
  effect of prior exposure of UG30 to 2,4-dinitrophenol (2,4-DNP) on degradation of ^{14}C-ring labeled 2,4-DNP, 198
  examples, 195
  examples of bacterial oxidative nitroaromatic transformation, 196*t*
  examples of reductive and partial reductive transformation by bacteria and fungi, 202*t*
  flavin monooxygenases in metabolism, 196–198
  GC–MS spectrum of trifluralin and its amino-derivative metabolite, 205*f*
  interest in modifying crop tolerance to diphenyl-ether herbicide acifluorfen, 213
  metabolism by oxidative or partial reductive processes, 206, 208
  metabolism of 2,4,6-trinitrotoluene (2,4,6-TNT) in terrestrial and aquatic plants, 211–212
  metabolism of 2,4-DNP initiated by *Sphingomonas* sp. UG30 PCP-flavin monooxygenase (pcpB), o-nitrophenol-monooxygenase, or by dioxygenase, 200*f*
  metabolism of pentachloronitrobenzene (PCNB) in cotton, 211
  microaerophilic metabolism of dinoseb by resting cell suspensions, 203, 206*t*
  microaerophilic metabolism of trifluralin by bacterial cell suspensions, 203, 204*t*
  microbial metabolic transformations of trifluralin resulting from initial aromatic nitroreduction, 207*f*
  monooxygenases for oxidative removal of nitro groups, 195–196
  nitroreduction of diphenylether herbicides, 202–203
  4-nitrotoluene as sole carbon and nitrogen source for *Pseudomonas* sp., 208
  3-nitrotoluene as sole carbon and nitrogen source for *Ralstonia eutropha* strain JMP134, 208
  other oxidative pathways, 199–201
  partial reductive nitroreductases, 201
  pathway for transformation of nitrobenzene initiated by partial nitroreduction in *P. pseudoalcaligenes* strain JS52, 208, 209*f*
  plant nitroaromatic transformations, 210–213
  potential for aromatic nitroreduction of dinitroaniline and dinitrophenol herbicides, 204

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ix002

  potential of several fungi to transform or mineralize 2,4,6-TNT, 210
  recovery of radioactivity from *Sphingomonas* sp. UG30 cultures treated with ^{14}C-2,4-DNP after 44 and 120 h, 199*t*
  reduction of nitrobenzene to phenylhydroxylamine by spinach, 212–213
  toluene dioxygenase metabolizing nitrotoluenes, 199–201
  transformation of trifluralin and dinoseb under aerobic and microaerophilic conditions, 203
  transformations of pentachloronitrophenol in peanut via aromatic nitroreduction or glutathione S-transferase mediated dechlorination or nitrite release, 211, 212*f*
Nitrobenzene
  biodegradation, 207–208
  reduction to phenylhydroxylamine by enzymes in spinach, 212–213
Nitroreduction. *See* Microbial reductive pathways
Nitrosation, conjugation reaction in bacteria, 111–112

O

Organohalides, agrochemical, reductive dehalogenation transformation in soil, 289*t*
Organophosphate insecticides
  hydrolysis by microorganisms, 76
  hydrolysis by plants, 75–76
  propanil hydrolysis inhibition, 73
  testing enzymatic hydrolysis, 184, 187
Organophosphonates
  2-amino-3-phosphonopropionic acid (phosphonoalanine), 148
  assaying carbon-phosphorus (C–P) lyase, 152
  biodegradation of glyphosate, 149–151
  biodegradation of organophosphonate insecticides, 155–157
  C–P bond, 145
  C–P lyase mediated biodegradation of synthetic phosphonates, 151–154
  catabolism of 2-aminoethanephosphonic acid (AEP, ciliatine), 146–147
  catabolism of naturally occurring, 148–149
  1,2-cis-epoxypropanephosphonic acid (phosphonomycin), 148
  conversion by microorganisms, 145–146
  glufosinate biodegradation, 149
  groups of bacteria metabolizing ciliatine, 147
  history of studies of phosphonate biodegradation, 146
  mechanism of dephosphonylation by C–P lyase in vivo, 153–154
  nerve agents, 156–157
  2-oxoalkanephosphonates degradation by bacteria and fungi, 147
  parathion degradation, 155–156
  phosphonoacetate biodegradation, 154–155
  phosphonyl intermediate, 152–153
  phosphoranyl intermediate, 153
  uptake and breakdown of phosphonates in *Escherichia coli*, 151
Oxadiazon, methyl conjugation by transformation by *Fusarium solani*, 106
Oxidative pathways. *See* Microbial oxidative pathways
Oxidative stress, corn glutathione S-transferases (GSTs), 262–263
Oxidoreductases, decontamination of water and soil, 189
Oxyfluorfen
  homogenization of treated corn seedlings, 256
  treating plants, 256
  See also Recombinant isoforms of glutathione S-transferase (GST) from corn
Oxygenolytic dehalogenation, mechanism, 285, 286*t*

P

Parathion
  biodegradation, 155–156
  decontamination by enzymes, 186*t*
  metabolic profiling, 46–47
  metabolic rates, 47*t*
  testing for bioremediation, 184, 185*t*
  See also Plant cell suspension cultures
Patent office, data types as synergism, 380–381
Pectin conjugates, linkage to chloroaniline, 122–123
Pentachloronitrobenzene (PCNB), metabolic reactions in plants, 211, 212*f*
Perchloromethylmercaptan, inhibition of esterases, 64

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ix002

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ix002

Peroxidase activity, use of plant materials containing for bioremediation, 190–191
Pesticide degradation, role of phosphatases and sulfatases, 77
Pesticide design, structure-activity relationships, 21
Pesticide development
  environmental effect on pesticide metabolism in plants, 13
  examples of herbicide/pesticide interactions in plants, 21*t*
  herbicide resistant crops, 10
  managing herbicide resistance in weeds, 9–10
  mechanisms of herbicide resistance, 9*t*
  pesticide safeners, antagonists, and synergists, 10–13
  pesticide selectivity, 8
  site of action, 8
Pesticide fate
  accelerated biodegradation, 14–15
  bioremediation, 15
  environment, 13–16
  phytoremediation, 16
Pesticide metabolism in plants and microorganisms
  accelerated biodegradation, 14–15
  aerobic metabolism under anoxic conditions using alternate terminal electron acceptors, 6–7
  antibody technology, 19–21
  approaches to herbicide discovery, 18
  bioremediation, 15
  carbon nutrition, 4–5
  chemical analytical techniques, 18–19
  cometabolism, 3–4
  complete xenobiotic mineralization, 6
  cytological techniques, 21
  environmental effects of pesticide metabolism in plants, 13
  enzymatic basis of pesticide transformation, 3–7
  examples of ELISA assays for common pesticides, 20*t*
  examples of herbicide/pesticide interactions in plants, 12*t*
  examples of pesticide mineralization by pure cultures of bacteria and fungi, 5*t*
  expression of all enzymes in metabolic pathway, 7
  extraction and purification methods for enzymes and other proteins, 18–19
  fundamental differences between plant and microbial metabolism, 4*t*
  genomics, 17–18
  herbicide resistant crops, 10
  integration of metabolic pathways, 7
  managing herbicide resistance in weeds, 9–10
  mechanisms of herbicide resistance, 9*t*
  mineralization, 5–7
  molecular biology techniques, 16–17
  oxidative metabolism of aromatic compounds in plants, 6
  pesticide fate in environment, 13–16
  pesticide safeners, antagonists, and synergists, 10–13
  pesticide selectivity, 8
  phytoremediation, 16
  ring cleavage for mineralization of aromatic pesticides, 6
  site of action, 8
  structure-activity relationships and pesticide design, 21
  technology and methodology, 16–21
  traditional analytical methodology and instrumentation, 19
  transgenic plants for expression of foreign proteins, 20–21
  xenobiotic degradation, 14
  See also Microorganism enzymes; Plant enzymes
Pesticide residues. *See* Bound pesticidal residues in crop plants
Pesticide selectivity, dependence on factors, 8
Pesticides
  cometabolism, 3–4
  discovery, 8
  enzymatic basis of transformation, 3–7
  extensive degradation in plants and environment, 3
  metabolic profiling using plant cell suspension cultures, 52*t*
  metabolic rates in wheat and soybean cell suspension cultures, 45*t*
  objectives of metabolism studies, 40–41
  transforming agricultural production, 2–3
Phanerochaete chrysosporium, model system for cytochrome P-450s, 178
Phase II metabolism
  herbicides, 317
  mechanisms of herbicide safeners in plants, 328*t*

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ix002

Phase I metabolism
  herbicides, 315
  mechanisms of herbicide safeners in plants, 328*t*
Phenolics
  structurally diverse biosynthetic compounds in nature, 333–334
  See also Cometabolism of xenobiotics
Phenoxyacetic acid, linkage to carbohydrate polymers, 122–123
Phenylcarbamates
  reaction pathway, 185*f*
  testing for bioremediation, 184
Phenylurea, P-450 genes in metabolism, 173
Phosphatases, role in pesticide degradation, 77
Phosphinothricin, catabolism, 149
Phosphinothricin (PPT)
  cloning PPT-resistant gene, 80
  metabolite for major commercial herbicides, 78, 79*f*
Phosphonates. *See* Organophosphonates
Phosphonoacetate, biodegradation, 154–155
Phosphonoalanine, catabolism, 148
Phosphonomycin, catabolism, 148
Phosphonyl intermediate, C–P lyase mediated biodegradation, 152–153
Phosphoranyl intermediate, C–P lyase mediated biodegradation, 153
Photoreductions
  electron donors for abiotic reductions in environment, 398
  environmental decomposition mechanism, 397–398
  primary carfentrazone-ethyl decomposition route in California ride field water and soil, 398, 401*f*
  See also Chloropropionic acid photoreduction
Phytoremediation, pesticide fate, 16
Picloram metabolism
  conjugation reactions by bacteria, 100–101
  differential conjugation affecting tolerance of plant, 100–101
  experiments determining sensitivity differences, 101
  proposed mechanical scheme in leafy spurge, 100*f*
Plant breeding, metribuzin, 366
Plant cell suspension cultures
  determination of metabolic patterns–metabolic profiling, 45–46
  evaluation of metabolic rates by standardized metabolism test, 43–45
  factors affecting metabolism study results, 43
  maintenance and characterization, 41–42
  metabolic profile of parathion, atrazine, and pyrene using, 47*t*
  metabolic profiling experiments with atrazine, 47–49
  metabolic profiling of atrazine using, 48*f*
  metabolic profiling of glufosinate using, 51*t*
  metabolic profiling of parathion, 47*f*
  metabolic profiling of pesticides and xenobiotics using, 52*t*
  metabolic profiling of pyrene using, 49*f*
  metabolic profiling studies with glufosinate, 50–52
  metabolic rates and patterns of parathion, 46
  metabolic rates and patterns of pyrene, 49–50
  metabolic rates of pesticides in wheat and soybean cell suspension cultures after 48 h incubation, 45*t*
  metabolism of xenobiotics by heterotrophic, 42–43
  modification to test procedures for avoiding ambiguity, 44–45
  plant metabolic pathways of glufosinate and metabolic profiling using, 51*f*
  requiring exogenous supply of auxin, 42
Plant enzymes
  amide hydrolysis, 64–65, 67
  applying pesticides as carboxylic acid esters, 61*f*
  assessment of substrate chemical structure and aryl acylamidase activity, 65, 67
  carbamate hydrolysis, 74
  ester hydrolysis in, 59–62
  herbicides as esters improving absorption into plant tissue, 60
  hydrolysis of naproanilide, 65
  hydrolysis of organophosphate insecticides, 75–76
  inhibition of plant amidases, 73
  nitrile hydrolysis, 77
  phytotoxicity of quinclorac herbicide derivatives, 62

- polycyclic alkanoic acid herbicides (PCAs), 60, 62
- resistance from intensive use of propanil, 65
- role of plant esterases in herbicidal activity in plants, 60*t*
- selectivity of sulfonylurea herbicides, 62
- structural examples of substituted amides, 66*f*
- use of materials containing peroxidase activity, 190–191
- *See also* Pesticide metabolism in plants and microorganisms
- Plant glutathione S-transferases (GSTs). *See* Glutathione S-transferases (GSTs)

Plant metabolism
- fundamental differences between, and microbial metabolism, 4*t*
- in vitro methods, 37
- microbial synthesis approach for predicting metabolites, 31–32

Plants
- environmental effects on pesticide metabolism in, 13
- *See also* Conjugation reactions in plants

Polychlorinated biphenyls (PCB)
- dechlorination in rhizosphere, 303
- degradation dependence on removal of inhibitory metabolites from chlorobenzoates, 344
- plant beneficial effects for enhancing biodegradation of chlorobenzoates, 344–345
- plant induction of PCB cometabolism, 343–346
- terpene enhanced PCB degradation, 345–346
- *See also* Cometabolism of xenobiotics

Polycyclic alkanoic acid (PCA) herbicides
- common herbicides, 60, 62
- hydrolysis in plants, 60, 62

Polycyclic aromatic hydrocarbon (PAH), rhizosphere, 343

Polymer chain reaction (PCR), molecular biology technique, 16–17

Pressurized liquid extraction (PLE), chemical analytical technique, 18

Profiling. *See* Metabolic profiling

Propanil
- ^{14}C-propanil metabolism by cell suspension of soil, water, and rhizosphere bacteria, 71*t*
- dissipation in soil by hydrolysis, 68
- hydrolysis inhibition, 73
- recovery of hydrolyzing isolates from Mississippi Delta, 70*t*
- resistance by intensive use, 65
- substrate specificity of enzyme preparations, 65, 67

Proteus mirabilis, bacterial glutathione S-transferase (GST), 243, 244*f*, 245*t*

Pyrene
- metabolic profiling, 49–50
- metabolic rates, 47*t*
- *See also* Plant cell suspension cultures

Pyridine herbicides
- controlling narrow-leaf and small seeded broad-leaf weeds, 129–130
- structures of representative, 130*f*
- *See also* Engineering plant tolerance to pyridine herbicides

Q

Quantitative-structure activity relationships (QSAR), methodology, 21

Quinclorac herbicide, comparing phytotoxicity of ester derivatives, 62

Quinone moieties, acting as terminal electron acceptors in rhizosphere, 295–296

Quinones. *See* Chloropropionic acid photoreduction

R

Radioactively labeled pesticides. *See* Bound pesticidal residues in crop plants

Random amplification polymorphic DNA (RAPD), molecular biology technique, 16–17

Recombinant isoforms of glutathione S-transferase (GST) from corn
- chemicals and statistics, 258
- classifications of cytosolic plant GSTs in crops, 254–255
- corn GSTs and oxidative stress, 262–263
- expression of cDNAs and protein purification under native conditions, 255
- formation of GST heterodimers in vitro, 260*t*
- glutathione peroxidase activities, 261–262
- GST assay method, 257

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ix002

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ix002

  - homogenization of oxyfluorfen-treated corn seedlings, 256
  - immunological studies, 263, 266
  - methods for cDNA cloning and construction of plasmids, 255
  - molecular weight calculation by gel filtration, 257
  - oxyfluorfen-treated plants, 256
  - polyacrylamide gel electrophoresis (PAGE) method, 256
  - preparation of linolenic acid hydroperoxide, 257–258
  - production of antisera and Western-blotting method, 256–257
  - purification of GST II from corn, 256
  - recombinant GST isoforms, 258
  - SDS–PAGE analysis of GST cDNA expression in *E. coli* and purification of recombinant corn GST isoforms, 259*f*
  - SDS–PAGE of proteins, 264*f*
  - specific activities, 262
  - substrate specificities of purified recombinant corn GST isoforms, 261*t*
  - substrate specificities of recombinant GST isoenzymes, 260–261
  - Western-blotting using GST26-antiserum, 264*f*
  - Western-blotting using GST27- and GST29-antiserum, 265*f*

Reduced uptake, theory of safener action, 323–324

Reductive dehalogenation
  - bioavailability of contaminants in rhizosphere, 298–300
  - biodegradation mechanism and pollutant examples, 284
  - cometabolic transformation, 288, 290
  - common agrochemical organohalides undergoing reductive dehalogenation (RED) transformation in soil, 289*t*
  - denitrifiers, 296
  - electron acceptors, 295
  - electron donors and nutrients in rhizosphere, 297–298
  - evidence of microbial RED in rhizospheric environments, 300, 303
  - examples of microbial RED in rhizospheric environment, 302*t*
  - fortuitous RDE through scavenging 'hot' electrons, 296
  - future directions, 303–304
  - halorespiration by specific enzymes, 293
  - halo-respiring microorganisms in rhizosphere, 297
  - hydrophobic contaminant bioavailability in SOM, 299
  - hypothetical scheme linking RDE to tetrachloroethene to methanogenesis, 290
  - mechanism, 285, 286t, 288
  - mechanism of energy conservation through halorespiration, 293
  - mechanism of physical protection of soil organic matter (SOM), 298–299
  - microorganisms capable of humic constituent respiration, 297
  - oxygen concentration and corresponding redox potential in rhizosphere, 294–295
  - physico-chemical factors for microbial RDE, 293–294
  - polychlorinated biphenyl (PCB) dechlorination in rhizosphere, 303
  - pure bacterial cultures with capability to respire organohalides, 292*t*
  - quinone moieties as terminal electron acceptors, 295–296
  - relationship between direct energetic metabolism and cometabolic dehalogenation in microorganisms, 291*f*
  - rhizosphere of terrestrial plants, 295
  - rhizosphere of terrestrial plants affecting bioavailability of organohalides in environment, 299–300
  - rhizosphere of wetland plants, 294–295
  - suitability of terrestrial and wetland rhizosphere environments to RED reactions, 300, 301*t*
  - terminal electron acceptor processes (TEAP), 296
  - thermodynamic considerations, 290, 293
  - trichloroethylene (TCE) concentrations and degradation, 300, 303
  - two-electron transfer reactions, 288

Reductive dehalogenation of chlorinated hydroquinones, catalysis by bacterial glutathione S-transferase (GST), 246*f*, 247

Reductive pathways. *See* Microbial reductive pathways

Residues in crop plants. *See* Bound pesticidal residues in crop plants

Resistance, genetic engineering of crops for bialaphos/glufosinate, 78–80

Resistance management, effect of $ynergy on, 386
Restriction fragment length polymorphism (RFLP), molecular biology technique, 16–17
Rhizosphere
  anaerobic and aerobic compartments coexisting in wetland plants, 294–295
  bioavailability of contaminants, 298–300
  degrader organisms, 334
  effects on biodegradation of xenobiotics, 336
  electron donors and nutrients, 297–298
  examples of microbial reductive environment in rhizospheric environments, 302*t*
  halo-respiring microorganisms, 297
  microbial degradation of halogenated compounds, 284
  microorganisms capable of humic constituent respiration, 297
  oxygen concentration and redox potential, 294
  plant categories, 294
  polycyclic aromatic hydrocarbon (PAH) degradation, 343
  quinone moieties acting as terminal electron acceptors, 295–296
  suitability of terrestrial and wetland, environments to reductive dehalogenation, 301*t*
  terrestrial plants, 295
  terrestrial plants affecting bioavailability of organohalides, 299–300
  See also Cometabolism of xenobiotics; Reductive dehalogenation
Rhizosphere effect, 336
Rice, glutathione S-transferase (GST) isozymes in plants, 222
Risk management, $ynergy and, 385–386

S

Safeners
  affecting other enzymes, 322–323
  antagonism and altered uptake/translocation as mechanism of action, 323–324
  benefits, uses, and structures, 311
  chemical structures of, enhancing glutathione S-transferase (GST) activity, 230*f*
  concentrations of GSH and cysteine in shoots of several grass species, 325*t*
  cytochrome P-450 based hydroxylation, 316
  definition, 228
  dichlormid, 310–311
  differential effects of safeners on herbicide metabolism in crops and weeds, 324–325
  discovery of safener binding protein in maize, 326–327
  effects on phase II metabolism of herbicides, 317–323
  effects on phase I metabolism of herbicides, 315–317
  elevating enzyme catalyzed reactions of xenobiotics in plants, 314*f*
  enhancing glutathione formation, 317–318
  enhancing glutathione S-transferase (GST) activity for herbicides in plants, 319–320
  examples of chemical structures of common types of commercial herbicide, 313*t*
  exploiting metabolic differences between crops and weeds, 327
  glutathione conjugation, oxidative stress, and herbicide metabolism in plants, 317
  glutathione-mediated reactions, 228–229
  improving selectivity and tolerance of herbicide, 10–11
  inducing vacuolar transporter activity, 320–322
  induction of P-450 monooxygenases, 315
  mechanisms of herbicide, in plants, 328*t*
  molecular approaches in research, 325–327
  1,8-naphthalic anhydride (NA) first commercial, 310
  non-enzymatic conjugation with glutathione (GSH) in plants, 318–319
  possible herbicide/safener combinations and uses, 312*f*
  safener BAS 145138 partially protecting maize from chlorimuron-ethyl injury, 322*f*
  site of action, 321–322
  species-specific cytochrome P-450s, 316–317

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ix002

  three general theories explaining possible mechanisms, 311, 313–314
  use, 11
Salicylic acid
  precise mechanism and role in induced systemic resistance, 340–341
  role in plant defense against pathogen invasion, 340
  salicylate inducible toluene monooxygenase enzyme, 341
  structure, 339*f*
  See also Cometabolism of xenobiotics
Sarin, degradation, 156–157
Selectivity
  conjugation imparting, 99–101
  pesticide, 8
Serendipity, pesticide discovery, 8
Simazine, degrading bacteria, substrates, and products, 271*t*
Sodium sulfide. *See* Chloropropionic acid photoreduction
Soil
  bioremediation involving lindane, 179
  coupling reaction with bentazon, 108
  humification of bentazon catalyzed by laccase of *Polyporus pinsitus*, 109*f*
  microbial synthesis approach for predicting metabolites, 31–32
Soil microbes, use for synthesis of potential metabolites of agrochemicals, 31
Soman, degradation, 156
Soybeans, glutathione S-transferase (GST) genes, 225
Split applications
  economics of $ynergy, 388–389
  influence on climate, 387
  mechanisms, 387–388
Structure-activity relationships, pesticide design, 21
Sulfatases, role in pesticide degradation, 77
Sulfonylurea herbicides
  detoxification of nicosulfuron, 359
  differential routes of metabolism of DPX-L8747 in crops and grass weeds, 361*f*
  differential routes to metabolism of chloroamben in tolerant and sensitive plants, 363*f*
  effect of structural substituents and application rates of nicosulfuron analogs on maize injury, 364*t*
  ester hydrolysis in plants, 62
  metabolic fate dependence on functional groups in both pyrimidine ring and aromatic ring, 359
  metabolic handles of sulfonylureas in different plant species, 362*f*
  metabolic pathway of nicosulfuron in maize, 362*f*
  possible herbicide/safener combinations and uses, 312*f*
Sulfoxidized thiocarbamate herbicides. *See* Thiocarbamate, sulfoxidized herbicides
Supercritical fluid extraction (SFE), chemical analytical technique, 18
Synergists
  examples of herbicide/pesticide interactions in plants, 11, 12*t*
  glutathione-mediated reactions, 229, 231
Synergy
  advantage of crop rotation, 377
  advantages of $ynergy, 384–385
  biological considerations and $ynergy, 383–384
  case histories of $ynergy, 386–392
  concept of using chemical mixtures, 376–377
  cost-effective crop production goal of farmers, 377–378
  cost of using multiple pest control strategies in concert, 378
  economics of synergy from mechanical strategies, 390–392
  economics of synergy from split applications, 388–389
  effect of $ynergy on resistance management, 386
  environmental considerations and $ynergy, 383
  influence of climate on split applications, 387
  interactions between chemicals and agronomic practices, 377
  legal, 380–381
  mathematical, 381, 383
  mathematics of measuring, 377
  mechanisms for split applications, 387–388
  pest-based, classical definitions, 378–383
  proposed new definition of $ynergy, 383–384
  risk and mechanical strategies, 390
  synergy and single pest, 379–380
  synergy versus $ynergy, 384–386
  $ynergy and risk management, 385–386
  $ynergy from crop rotations, 392

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ix002

$ynergy from herbicide/cultivation interactions, 389–392
  $ynergy from split applications of herbicides, 387–389
  types, 378–379

T

Terbuthylazine, degrading bacteria, substrates, and products, 271*t*
Terpenes
  enhancing polychlorinated biphenyl (PCB) degradation, 345–346
  enrichment of xenobiotic degraders on, 346
  structurally diverse biosynthetic compounds in nature, 333–334
  See also Cometabolism of xenobiotics
Terpenoids, plant
  biodegradation of camphor in *Mycobacterium rhodochrous*, 338
  biodegradation of monoterpenes, 338
  essential, 337
  function, 337–338
  research, 338, 340
  See also Cometabolism of xenobiotics
Thiadiazolidin-one herbicides, activity of glutathione S-transferases towards, 221–222
Thiazopyr
  assay method for tolerance, 133
  assays for tolerance in tobacco and tomato, 142
  confirmation as target of deactivation, 134
  custom synthesis, 130
  dose titration under pre-plant incorporation in control and transgenic tomato seeds, 143*f*
  engineering plant tolerance, 130
  identification of deactivation pathway, 134–136
  metabolism studies in animals and plants, 130
  plant tolerance by metabolic deactivation mechanism, 129–130
  resistance of transformed tobacco and tomato plants, 64
  structure, 130*f*
  time course of hydrolysis of pyridine herbicides by porcine liver esterases, 136*f*
  time course of oxidation of pyridine herbicides by NADPH-fortified rat liver microsomes, 135*f*
  See also Engineering plant tolerance to pyridine herbicides; Pyridine herbicides
Thiocarbamate, sulfoxidized herbicides
  herbicides conjugating with glutathione, 226
  safeners enhancing glutathione conjugation, 229
Thiocarbamates, hydrolysis in microorganisms, 75
Thiolytic dehalogenation, mechanism, 285, 286*t*
Tolerance
  chemical regulation of metabolism-based, by herbicide synergists and safeners, 228
  plant breeding for herbicide, 366
  See also Designing herbicide tolerance; Engineering plant tolerance to pyridine herbicides
Toluene dioxygenase, metabolism of nitrotoluenes, 199–201
Tomato, metribuzin metabolism, 102
Toxicology, bound pesticidal residues in plants, 125–126
Transgenic plants, expression of foreign proteins, 20–21
Translocation, altered, action of safeners, 323
s-Triazine herbicides
  chemical structures of several, 274*f*
  evolutionary aspects of microbial degradation, 276–278
  genetics of degradation, 272
  nitrosation in soil under nitrifying conditions, 111–112
  some s-triazine degrading bacteria, substrates, and degradation products, 271*t*
  See also Atrazine
Triazophos, enzymatic hydrolysis rates, 185*t*
Trichloroethylene, reductive dehalogenation, 300, 303
Tridiphane
  chemical structure of, and GS-tridiphane conjugate, 232*f*
  synergist and glutathione-mediated reactions, 229, 231
2,4,6-Trinitrotoluene
  fungi with potential to transform or mineralize, 210
  hydrogenation, 209
  metabolism in terrestrial and aquatic plants, 211–212

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ix002

U

Uptake, reduced
  mechanisms of herbicide safeners in plants, 328*t*
  theory of safener action, 323–324
Urea herbicides, possible herbicide/safener combinations and uses, 312*f*

V

Vacuolar compartmentalization, immobilization of pesticides, 98
Vacuolar transporter activity, safener induction, 320–322

W

Wheat
  glutathione S-transferase (GST) isozymes in plants, 222
  pathogen-induced gene encoding GST, 225

X

Xenobiotic metabolism, biochemistry and role of plant esterases, 59
Xenobiotics
  acylation by fungi, 107
  benefits of using plants to assist biodegradation, 334
  conjugation, 90
  detoxification by glutathione conjugation, 226, 240–241
  enrichment of xenobiotic degraders on terpenes, 346
  enzyme catalyzed reactions of, in plants, 314*f*
  enzymes showing ability to degrade, 187
  influence of plants on microbial metabolism, 284
  metabolic profiling using plant cell suspension cultures, 52*t*
  metabolism by heterotrophic plant cell cultures, 42–43
  metabolism by microbial species, 31
  metabolism studies using heterotrophic cell suspensions, 41–42
  objectives of metabolism studies, 40–41
  plant metabolism, 41
  significance of conjugation by fungi, 108
  See also Bacterial glutathione S-transferases; Cometabolism of xenobiotics; Metabolic profiling; Nitroaromatic pesticides
  Xenobiotics, detoxification. *See* Bacterial glutathione S-transferases
Xylosylation, conjugation reactions in fungi, 103–104

Downloaded by 89.163.34.136 on September 17, 2012 | http://pubs.acs.org
Publication Date: December 1, 2000 | doi: 10.1021/bk-2001-0777.ix002